Metabolic Engineering of the Chloroplast Genome Using the *Echerichia coli ubi*C Gene Reveals That Chorismate Is a Readily Abundant Plant Precursor for p-Hydroxybenzoic Acid Biosynthesis[1]

Paul V. Viitanen, Andrew L. Devine, Muhammad Sarwar Khan[2], Deborah L. Deuel, Drew E. Van Dyk, and Henry Daniell*

DuPont Experimental Station, Wilmington, Delaware 19880–0402 (P.V.V., D.L.D., D.E.V.D.); and Department of Molecular and Microbiology, University of Central Florida, Orlando, Florida 32816–2360 (A.L.D., M.S.K., H.D.)

p-Hydroxybenzoic acid (pHBA) is the major monomer in liquid crystal polymers. In this study, the *Escherichia coli ubi*C gene that codes for chorismate pyruvate-lyase (CPL) was integrated into the tobacco (*Nicotiana tabacum*) chloroplast genome under the control of the light-regulated *psb*A 5′ untranslated region. CPL catalyzes the direct conversion of chorismate, an important branch point intermediate in the shikimate pathway that is exclusively synthesized in plastids, to pHBA and pyruvate. The leaf content of pHBA glucose conjugates in fully mature T_1 plants exposed to continuous light (total pooled material) varied between 13% and 18% dry weight, while the oldest leaves had levels as high as 26.5% dry weight. The latter value is 50-fold higher than the best value reported for nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL. Despite the massive diversion of chorismate to pHBA, the plastid-transformed plants and control plants were indistinguishable. The highest CPL enzyme activity in pooled leaf material from adult T_1 plants was 50,783 pkat/mg of protein, which is equivalent to approximately 35% of the total soluble protein and approximately 250 times higher than the highest reported value for nuclear transformation. These experiments demonstrate that the current limitation for pHBA production in nuclear-transformed plants is CPL enzyme activity, and that the process becomes substrate-limited only when the enzyme is present at very high levels in the compartment of interest, such as the case with plastid transformation. Integration of CPL into the chloroplast genome provides a dramatic demonstration of the high-flux potential of the shikimate pathway for chorismate biosynthesis, and could prove to be a cost-effective route to pHBA. Moreover, exploiting this strategy to create an artificial metabolic sink for chorismate could provide new insight on regulation of the plant shikimate pathway and its complex interactions with downstream branches of secondary metabolism, which is currently poorly understood.

All plants normally produce p-hydroxybenzoic acid (pHBA), albeit usually in small quantities. Radioisotope studies with *Lithospermum erythrorhizon* suggest that this compound is derived from the CoA ester of p-hydroxycinnamic acid (pHCA-CoA) through a β-oxidation-like mechanism (Loscher and Heide, 1994). However, earlier studies with the same plant species (Yazaki et al., 1991) and carrot (*Daucus carota*) cell cultures (Schnitzler et al., 1992) supported a cleavage mechanism that occurs via intermediacy of p-hydroxybenzaldehyde. Notwithstanding our current ignorance of the detailed plant biosynthetic pathway, a number of studies have shown that it is possible to dramatically elevate pHBA levels in plants through metabolic engineering. Indeed, two different ''single-enzyme'' pathways have been described, both involving microbial proteins that have no known plant counterparts. One of these enzymes is chorismate pyruvate-lyase (CPL). Its substrate is chorismate, an important branch point intermediate in the shikimate pathway, which is largely, if not entirely, synthesized in chloroplasts and other types of plastids (Hrazdina and Jensen, 1992; Herrmann and Weaver, 1999). Using nuclear transformation and a chloroplast-targeted version of *Escherichia coli* CPL (TP-UbiC), Heide and coworkers were able to generate transgenic tobacco plants that had leaf levels of pHBA that were 3 to 4 orders of magnitude greater than wild-type plants (Siebert et al., 1996). Virtually all of the compound (>98%) accumulated in the vacuole as two Glc conjugates, a phenolic glucoside and a Glc ester, which both contained a single Glc molecule attached to the aromatic hydroxyl or carboxyl group of pHBA by a 1-*O*-β-D linkage. Glc conjugation, which takes place in the cytosol via distinct UDP-glucosyltransferases (Lim et al., 2002), is required for vacuolar uptake, and recent studies have shown that the pHBA phenolic glucoside and Glc ester are

[1] This work was supported in part by the National Institutes of Health (grant no. R 01 GM63879) and by the U.S. Department of Agriculture (grant no. 3611–21000–017–00D to H.D.).

[2] Present address: National Institute of Biotechnology and Genetic Engineering, Jhang Road, Faisalabad, Pakistan.

* Corresponding author; e-mail daniell@mail.ucf.edu; fax 407–823–0956.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.050054.

transported by separate carriers that have different mechanisms (Bartholomew et al., 2002). The highest leaf content of pHBA Glc conjugates in nuclear-transformed tobacco (*Nicotiana tabacum*) plants was 0.52% dry weight, which is equivalent to 0.24% free pHBA after correcting for the mass of the attached Glc residue. Nevertheless, the CPL-expressing tobacco plants were perfectly healthy and phenotypically indistinguishable from nontransformed control plants (Siebert et al., 1996). TP-UbiC was subsequently expressed in tobacco cell cultures (Sommer and Heide, 1998; Sommer et al., 1998) and hairy root cultures of *Lithospermum erythrorhizon* (Sommer et al., 1999), and the results were remarkably similar. In none of these studies, however, did the leaf content of pHBA Glc conjugates exceed 1.0% dry weight. Although these experiments are very encouraging and constitute a major breakthrough for plant metabolic engineering, a commercially viable pathway for pHBA production in plants will require 10- to 20-fold higher levels of product accumulation.

The other microbial protein that has been used to elevate pHBA levels in tobacco is the 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) of *Pseudomonas fluorescens* (Mayer et al., 2001). This enzyme catalyzes a sequential hydration and retro-aldol cleavage of p-hydroxycinnamoyl-CoA (pHCA-CoA), a key intermediate in the phenylpropanoid pathway, to yield p-hydroxybenzaldehyde (Gasson et al., 1998; Mitra et al., 1999). In tobacco, the vast majority of the aldehyde was oxidized by endogenous plant enzymes that remain to be elucidated, and the metabolic fate of the resulting pHBA was glucosylation and vacuolar uptake similar to the situation with CPL. The HCHL route to pHBA production in plants is technically less complicated than the CPL pathway from a metabolic engineering perspective since pHCA-CoA is synthesized in the cytosol. Hence, there is no need to target the foreign protein across a lipid bilayer into an intracellular organelle to gain access to its substrate. In HCHL-expressing tobacco plants, pHBA Glc conjugates in leaf tissue accumulated to approximately 2.9% dry weight, which translates to approximately 1.3% nonglucosylated pHBA (Mayer et al., 2001). Although this represents a 5- to 6-fold improvement over CPL-mediated pHBA production, the HCHL-expressing tobacco plants suffered a severe depletion of phenylpropanoids that resulted in numerous phenotypic abnormalities, including leaf chlorosis, stunted growth, male sterility, and altered lignin content. Clearly, the ability of these plants to replenish pHCA-CoA could not keep pace with the massive diversion of this compound to pHBA Glc conjugates. These results strongly suggest that substrate availability, not enzyme activity, sets an upper threshold on pHBA accumulation in tobacco plants that hyperexpress HCHL, at least in leaf tissue.

Although it is conceivable that the current limitation for CPL-mediated pHBA production in plants is carbon flux through the plastid shikimate pathway, other explanations seem more likely. One potential limitation is the low levels of transgene expression caused by position effect due to random integration of transgenes or gene silencing in nuclear transgenic lines (Voinnet, 2001). Another obvious limitation of nuclear transformation is the poorly understood phenomenon of cosuppression, which could set an upper limit on CPL enzyme activity. Another potential area for improvement may lie in the design of a better chloroplast targeting sequence to achieve higher levels of enzyme activity in the intracellular compartment of interest. Indeed, there was a positive correlation between CPL specific activity and accumulation of pHBA Glc conjugates in several of the studies cited above. Furthermore, in none of these studies was there any indication that saturation had been achieved with respect to enzyme. Most naturally occurring chloroplast proteins are nuclear-encoded and synthesized on cytosolic ribosomes as larger M_r precursors with a cleavable N-terminal transit peptide. Following chloroplast protein import, the transit peptide is proteolytically removed to yield the mature polypeptide. Although hundreds of transit peptide sequences are now known, our ability to manipulate them to achieve optimal chloroplast targeting of a foreign protein is, at best, still a matter of trial and error. Simply attaching a chloroplast transit peptide to the N terminus of the passenger protein does not guarantee success. Even very subtle changes in the vicinity of the natural cleavage site of the Rubisco small subunit precursor can lead to diminished chloroplast uptake (Wasmann et al., 1988) and/or aberrant proteolytic processing (Robinson and Ellis, 1984, 1985).

It is occasionally observed that chloroplast uptake of foreign proteins can be improved by including a small portion of the mature N terminus of the transit peptide donor in addition to the transit peptide and scissile bond (Schreier et al., 1985; Van den Broeck et al., 1985). However, this approach is still associated with a high degree of unpredictability that is inextricably linked to the passenger protein. For example, in an attempt to improve plant pHBA production, Heide's group fused the Rubisco small subunit transit peptide and first 21 amino acid residues of the mature polypeptide to the N terminus of *E. coli* CPL (Sommer and Heide, 1998; Sommer et al., 1998). Surprisingly, however, this manipulation resulted in much lower levels of pHBA than the artificial fusion protein that was used in the earlier studies, TP-UbiC. One of the obvious pitfalls of this strategy is that cleavage of the transit peptide results in a CPL variant with an unnatural N-terminal extension that could have detrimental effects on catalytic activity and/or enzyme stability in the chloroplast compartment.

An alternate approach to express foreign proteins or enzymes that function within chloroplasts would be to directly integrate and express transgenes via the chloroplast genome. Such an approach has additional advantages including high levels of transgene expression (Daniell et al., 2002; Devine and Daniell, 2004),

transgene containment (Daniell, 2002), and multigene engineering (DeCosa et al., 2001; Daniell and Dhingra, 2002; Lossl et al., 2003; Ruiz et al., 2003). Moreover, the chloroplast is an ideal compartment to accumulate certain proteins or their biosynthetic products that would be harmful if they were accumulated in the cytoplasm (Daniell et al., 2001a; Lee et al., 2003; Leelavathi et al., 2003; Daniell et al., 2004a). Chloroplast transformation also eliminates positional effects that are frequently observed with nuclear transformation and no gene silencing has been observed so far at the level of transcription (Lee et al., 2003) or translation (DeCosa et al., 2001). Consequently, independent chloroplast transgenic lines have very similar levels of foreign gene expression and there is no need to screen hundreds of transgenic events. 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., 2004b, 2004c), vaccines for human (Daniell et al., 2001a, 2004c; Daniell, 2004; Watson et al., 2004) or animal use (Molina et al., 2004), and biomaterials (Guda et al., 2000; Lossl et al., 2003). Although most of these studies were done in tobacco, highly efficient stable plastid transformation of major crop species has recently been reported for carrot (Kumar et al., 2004a), cotton (*Gossypium hirsutum*; Kumar et al., 2004b), and soybean (*Glycine max*; Dufourmantel et al., 2004).

This study is the first attempt to utilize chloroplast transformation for metabolic engineering to generate plants that accumulate large amounts of a small aromatic compound of significant commercial value, pHBA. Towards this goal we have stably integrated the unmodified *E. coli ubi*C gene into the tobacco chloroplast genome and studied the consequences of hyperexpression of this enzyme in leaf and stem tissue. Another distinguishing feature of this work is that the pHBA levels reported are based on separately processed total leaf and total stalk material that was obtained from fully mature first- and second-generation plastid-transformed plants. It should also be emphasized that this is the first time that stalk production of pHBA has been examined in either CPL- or HCHL-expressing tobacco plants. Finally, our experiments provide unequivocal evidence that the current limitation for CPL-mediated pHBA production in nuclear-transformed plants is achieving high enough levels of enzyme activity in the chloroplast compartment, the site of chorismate synthesis, and that this obstacle is easily circumvented using plastid transformation. Until now high-level production of pHBA in plants has been elusive.

RESULTS

Tobacco chloroplast transformation vector pLD-CtV previously developed in the Daniell laboratory (Daniell et al., 1998, 2001a; Guda et al., 2000; DeCosa et al., 2001) was used to integrate the *E. coli ubi*C gene between the *trnI* and *trnA* genes that act as the flanking sequences to facilitate homologous recombination into the inverted repeat region of the tobacco chloroplast genome. The chloroplast transformation vector pLDK, which contains the chorismate pyruvate-lyase gene (*ubi*C), was expressed under the control of the *psb*A 5′ untranslated region (UTR)/promoter to maximize expression. It has previously been shown that genes under the control of the *psb*A promoter/5′UTR achieve very high levels of expression (Fernandez San-Millan et al., 2003; Dhingra et al., 2004; Watson et al., 2004). The 5′UTR has been hypothesized to enhance translation of proteins under its control (Eibl et al., 1999). The *aad*A gene confers spectinomycin resistance for selection of transformed shoots (Goldschmidt-Clermont, 1991). The *psb*A 3′UTR located at the 3′ end of the *ubi*C gene confers transcript stability (Fig. 1A; Stern and Gruissem, 1987).

Chloroplast transgenic plants were obtained by particle bombardment as previously described (Daniell, 1997; Daniell et al., 2004d; Kumar and Daniell, 2004). More than 20 independent shoots tested positive for stable integration of CPL. Each shoot was subjected to several rounds of selection with spectinomycin (500 μg/mL). Nuclear transformants usually do not survive this high level of concentration of spectinomycin due to low levels of transgene expression and are therefore eliminated in the selection process. However, to identify undesirable spontaneous mutations that occur at the level of the 16S rRNA gene, a PCR strategy was employed. One pair of primers was designed to determine site-specific integration of CPL into the chloroplast genome by homologous recombination (Daniell et al., 2001b). The 3P primer lands on the native chloroplast genome, upstream of the site of integration. The second primer 3M lands on the *aad*A gene (selectable marker; Fig. 1A). The presence of a 1.65-kb PCR product confirms site-specific integration of transgenes into the chloroplast genome (Fig. 1B). Nuclear transformants and spontaneous mutants do not yield this PCR product because they lack the necessary combination of the *aad*A gene adjacent to chloroplast flanking sequence. Following the second round of selection, shoots were transferred to MSO rooting medium that contained 500 μg/mL spectinomycin.

Southern blotting was performed to confirm stable integration of transgenes into the chloroplast genome and to determine their homoplasmy or heteroplasmy. Upon achieving homoplasmy, all chloroplast genomes contain the integrated *ubi*C gene and are hence identical. In contrast, the presence of untransformed chloroplast genomes is a clear indication of heteroplasmy. Southern blots were probed with either a gene specific (*ubi*C) probe or a flanking sequence probe. The *ubi*C

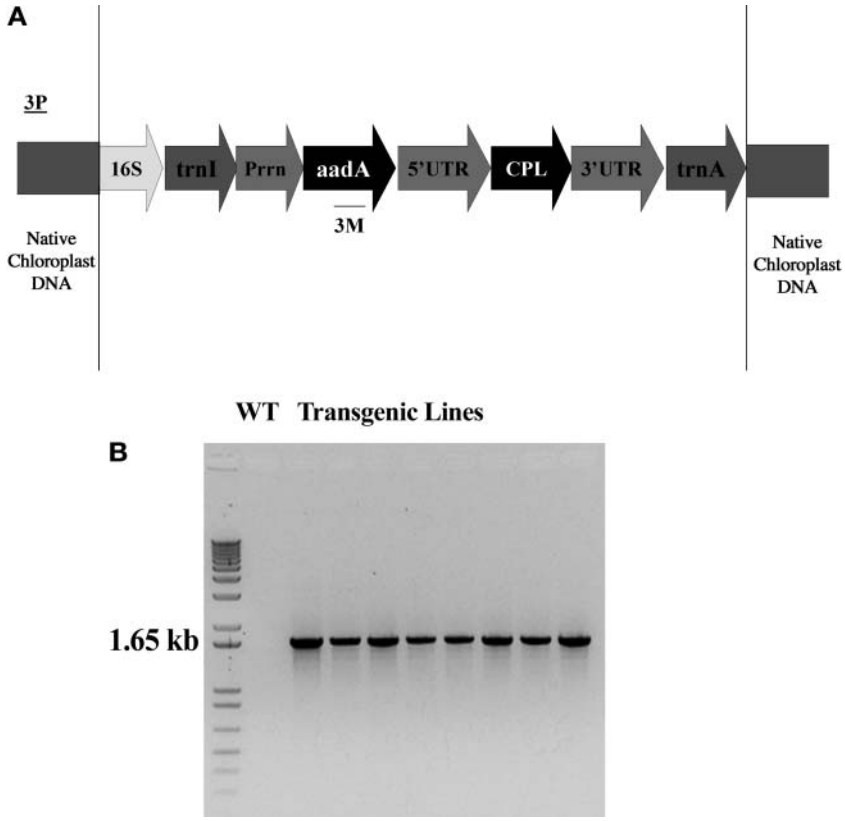

Figure 1. A, Schematic representation of gene cassette integrated into chloroplast genome. The map shows the gene cassette integrated into the chloroplast genome and PCR primers 3P/3M (expected fragment 1.65 kb) landing sites. These primers were used to screen out mutants and to confirm site-specific integration. Prrn, 16s RNA promoter; *aad*A: aminoglycoside 3′-adenylyl transferase; *psb*A 5′UTR; CPL, *ubi*C gene; *psb*A 3′UTR, terminator. B, PCR analysis of untransformed wild type and transformants. 3P3M PCR lanes: 1, MW; 2, untransformed; 3 to 4, T0 lines; 5 to 10, T1 lines. As expected, untransformed does not show 1.65-kb fragment. Transformed lines with 1.65-kb fragment indicate site-directed integration.

gene specific probe (0.3 kb) was obtained by *Sma*I/*Bam*HI digestion of the pLDK plasmid (Fig. 2A). The flanking sequence probe (0.81 kb) was obtained by *Bam*HI/*Bgl*II digestion of the pUC-ct plasmid, which contains the chloroplast flanking sequences *trnI* and *trnA* (Fig. 2A). The plant DNA was digested with *Afl*III (Fig. 2A). Upon hybridization with the flanking sequence probe transformed chloroplasts should exhibit a 6.3-kb fragment and untransformed chloroplasts a 4.2-kb fragment. If the 4.2-kb fragment is not seen in the transgenic line, this is an indication that all chloroplast genomes have been transformed and homoplasmy has been achieved, within the limit of detection. All transgenic lines tested positive for site-specific integration when hybridized with the *ubi*C probe and untransformed lines showed no such fragment (Fig. 2C). Most of the lines from T_1 whose seeds were germinated in the presence of spectinomycin showed only the 6.3-kb fragment, indicating again that homoplasmy had been achieved within the levels of detection and maintained in subsequent generations (Fig. 2D).

Shown in Figure 3 is the developmental time course for pHBA accumulation in leaves for a representative T_0 plant (line 4). Leaf punches obtained from the first or second leaf from the bottom of the plant were used for this analysis, and variation between replicates was typically less than 10%. Consistent with previous observations with nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL (Siebert et al., 1996), virtually all of the pHBA accumulated in the vacuole as Glc conjugates, a phenolic glucoside and Glc ester. Both compounds contain a single Glc molecule attached by a 1-*O*-β-D linkage and are readily converted to free pHBA by acid or base hydrolysis (Siebert et al., 1996). Also similar to nuclear-transformed tobacco plants expressing CPL, the phenolic glucoside was the predominant species in mature leaf tissue of the plastid-transformed plants, accounting for 60% to 80% of the total pHBA at all stages of development.

When grown under the normal light/dark cycle (16 h on/8 h off), the leaf content of pHBA Glc conjugates in the T_0 plant steadily increased over a 3-month period to a maximum value of 15% dry weight. However, there was further increase in product accumulation when the plant was shifted to continuous light (arrow in Fig. 3). This phenomenon, which was also observed with other T_0 and T_1 plants (data not shown), supports the notion that the *psb*A 5′ UTR is stimulated by light (Eibl et al., 1999; Daniell et al., 2004b; Dhingra et al., 2004; Watson et al., 2004). Indeed, under conditions of continuous light the level of pHBA Glc conjugates in old leaf tissue routinely approached approximately 25% dry weight (Figs. 3 and 4). To put this in perspective, the latter value corresponds to a pHBA content of approximately 11.5% dry weight after correcting for the attached

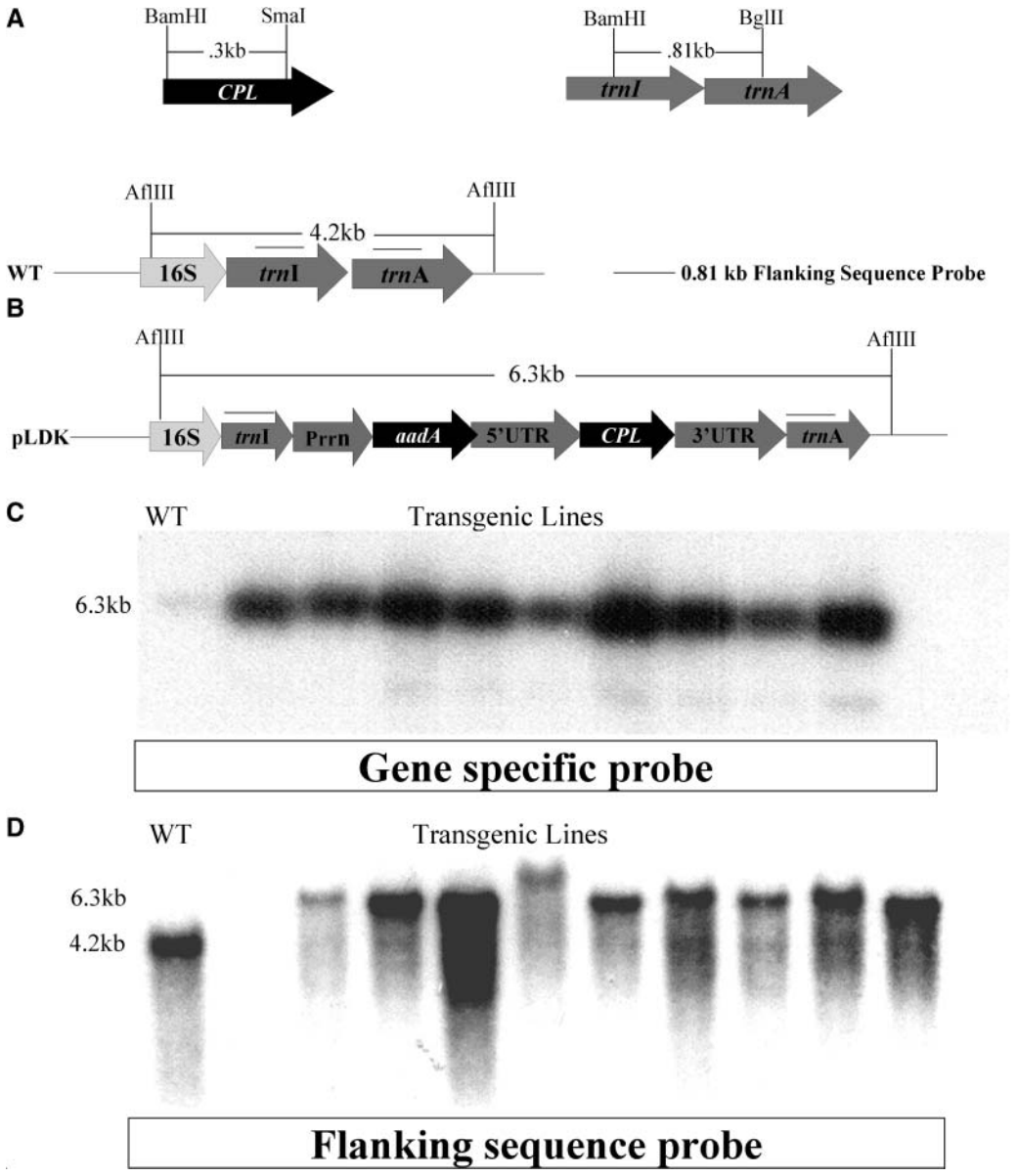

Figure 2. Southern-blot analysis to determine site-specific integration and homoplasmy. A, The 0.3-kb CPL specific fragment and 0.81-kb flanking sequence fragment that were used as probes for Southern-blot analysis. B, Untransformed tobacco plant DNA digested with *Afl*III (expected fragment size, 4.2 kb). Transformed tobacco plant DNA digested with *Afl*III (expected fragment size, 6.3 kb). Southern-blot analysis of untransformed and T_1 transformed lines. C, Lane 1, untransformed; lane 2, blank; lanes 3 to 5, T_1 lines-3 A to C; lanes 6 to 8, T_1 lines-4 A to C; lanes 9 to 11, T_1 lines-5 C, E, and F. Lane designations are the same for both blots except that there is no blank lane in C. C, Probed with CPL coding sequence. D, Probed with chloroplast DNA flanking sequence containing the *trnA* and *trnI* genes.

Glc moiety. More important, the leaf content of pHBA Glc conjugates in the plastid-transformed T_0 tobacco plants was about 50-fold higher than the best value reported for nuclear-transformed tobacco plants expressing a chloroplast-targeted version of CPL (Siebert et al., 1996).

Since product accumulation continued to increase over the span of several months, it was anticipated that young leaves would have a lower content of pHBA Glc conjugates than old leaves, and this turned out to be the case. As shown in Figure 4, the oldest leaves at the bottom of the plant had 5 to 6 times more pHBA than the youngest leaves at the top, while samples obtained from mature green leaves half way up the stalk contained intermediate levels. Although this experiment was performed with a T_1 plant that was derived from line 4, all the plastid-transformed plants exhibited this trend. It is also interesting to note that product accumulation in the first- and second-generation line 4 plants was very similar when old leaf tissue was

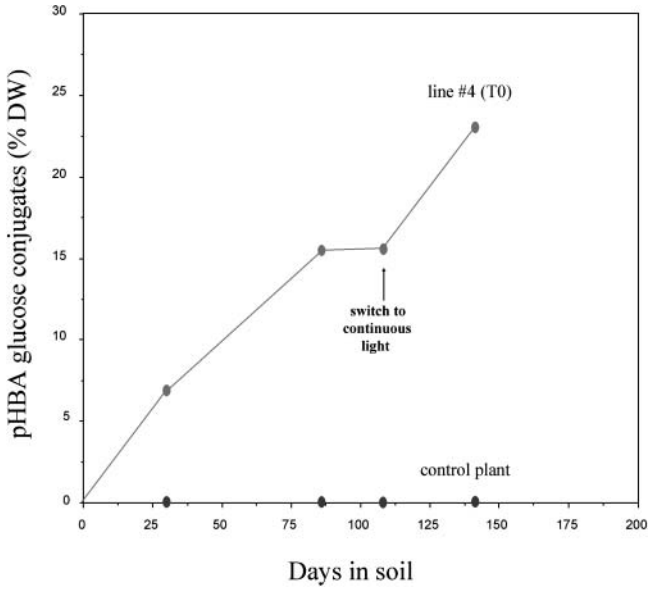

Figure 3. Developmental time course of pHBA accumulation in line 4 (a T_0 plant) and a nontransformed control plant. Values shown for pHBA Glc conjugates represent the sum of the phenolic glucoside and Glc ester and are expressed as a percentage of dry weight (% DW). For this analysis, leaf punches were obtained from the first or second leaf from the bottom of the plant (i.e. old leaves) at indicated times and the extracted tissue was analyzed by HPLC for Glc conjugates as described in ''Materials and Methods.'' The vertical arrow indicates when the plant was shifted to continuous light.

analyzed, reaching a maximum value of approximately 25% dry weight in both cases. This observation suggests that the line 4 T_0 plant was homoplasmic or nearly homoplasmic, and further support for this conclusion is shown in Table I. Regardless of the explanation, it is clear from the forgoing experiments that the *E. coli ubi*C gene integrated in the plastid genome was stably inherited in the second-generation plants.

Because the amount of pHBA in leaves depends on not only the age of the plant but also the age of the leaves, the leaf punch experiments described above provide no insight on perhaps the most important question from a biotechnology perspective: What is the total leaf content of pHBA in a fully mature plastid-transformed tobacco plant? It is also of interest to know how much product accumulates in the stalk when contemplating the commercial feasibility of a plant pHBA production platform. Surprisingly, there are no reports in the literature regarding pHBA levels in stem tissue for nuclear-transformed tobacco plants expressing either CPL or HCHL. To address these important questions, fully mature plastid-transformed tobacco plants were sacrificed and all biomass above the ground was harvested for analysis. Total leaf and total stalk material were segregated and the tissues were lyophilized to dryness and ground to a fine powder in an electrically driven mill. Following this procedure, the homogeneous dry plant material was analyzed by HPLC for pHBA Glc conjugates. The results from these experiments are summarized in Table I for selected first- and second-generation plastid-transformed plants.

The total leaf content of pHBA Glc conjugates for the three T_0 lines ranged from 10.9% dry weight to 15.2% dry weight. The mean value ($\pm$SE) was 13.15% $\pm$ 1.26% dry weight, which is roughly one-half the amount that was observed with leaf punches that were obtained from the oldest leaves on the plant. To determine the stability of the transgene and long-term effects on the health of the plant, four second-generation plants were subjected to the same analysis. Like the parental lines, the T_1 plants were not adversely affected by pHBA overproduction and were phenotypically indistinguishable from nontransformed control plants (Fig. 5). The two line 4 offspring had slightly lower levels of product accumulation than the T_0 plant, but these differences are probably not significant. On the other hand, there was a 50% to 70% increase in the total leaf content of pHBA Glc conjugates for the two line 3 descendants. The most logical explanation for this discrepancy is that the line 3 T_0 plant was heteroplasmic, while the T_1 plants were homoplasmic. Indeed, a mixed population of chloroplast genomes in T_0 plants is frequently observed with plastid transformation (Guda et al., 2000; Watson et al., 2004), but homoplasmy is almost always achieved by the second generation.

The data shown in Table I also reveals the dramatic difference between leaf and stalk content of pHBA, and all the first- and second-generation plants exhibited the same trend. The mean value for pHBA Glc conjugates in total stalk material representing fully mature plants was 2.3% dry weight $\pm$ 0.36% dry weight for the T_0 lines and 2.5% dry weight $\pm$ 0.05% dry weight for the T_1 lines. Thus, product accumulation in the stalk was 5- to 8-fold lower than corresponding leaf levels. Another notable difference between leaf and stalk production of pHBA was the Glc conjugate profile. In stem tissue, almost the entire compound was converted to the phenolic glucoside and only trace amounts of the Glc ester were detected (<5%).

In Arabidopsis (*Arabidopsis thaliana*), UDP-glucosyltransferases are members of a multigene family that consists of at least 107 distinct open reading frames (Li et al., 2001; Ross et al., 2002). Moreover, recent in vitro experiments with purified recombinant proteins have shown that eight of these open reading frames code for polypeptides that are able to glucosylate pHBA (Lim et al., 2002). Three of the enzymes only catalyze the formation of the phenolic glucoside, while the others exclusively attach Glc to the aromatic carboxyl group to form the Glc ester. These observations, coupled with the almost complete absence of the pHBA Glc ester in tobacco stem tissue and to a lesser degree leaf tissue, have very important implications for a plant-based production platform. Since the compound of interest is free pHBA and removing the Glc could be an expensive step in downstream processing, it was important

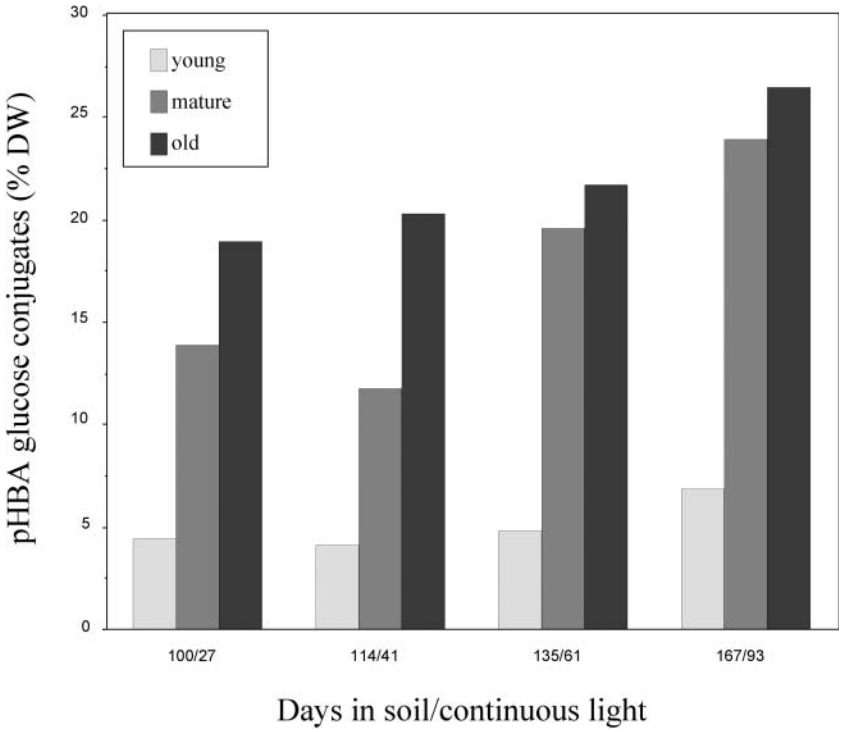

Figure 4. Levels of pHBA Glc conjugates in young, mature, and old leaf tissue at different stages of development. The plant analyzed for this experiment was line 4B (a T_1 plant that was derived from line 4). Values shown for pHBA Glc conjugates represent the sum of the phenolic glucoside and Glc ester in leaf punches and are expressed as a percentage of dry weight. The numbers below the four sets of bars indicate the age of the plant (left no.) and the number of days the plant was grown in continuous light (right no.) at the time of analysis.

to determine which Glc conjugate is easiest to hydrolyze.

Figure 6A shows the relative susceptibility of the pHBA phenolic glucoside and Glc ester to acid hydrolysis after 48 h at 60°C. From this data it is clear that the Glc ester is the most acid labile species. Quantitative conversion of this compound to free pHBA occurred with as little as 0.1 N HCl, while a 5-fold higher concentration of acid was required for complete hydrolysis of the phenolic glucoside. Since both Glc conjugates and free pHBA have limited water solubility at acid pH, alkaline hydrolysis was also examined (Fig. 6B). Again, the phenolic glucoside was relatively stable under the conditions employed and complete hydrolysis required >0.5 N NaOH. In marked contrast, 0.1 N NaOH was sufficient to release all of the pHBA from the Glc ester. Based on these observations, as well as economic and environmental considerations, we conclude that the Glc ester is the conjugate of choice to make in plants, if it is feasible to alter the in vivo partitioning of pHBA through metabolic engineering with an appropriate UDP-glucosyltransferase.

Table I. *pHBA Glc conjugate levels for total leaf and total stalk material are shown for selected T_0 and T_1 lines*

Values shown for pHBA Glc conjugates represent the sum of the phenolic glucoside and Glc ester and are expressed as a percentage of dry weight. Fully mature tobacco plants were used for this experiment, and all material above the ground was included in the analysis. The plants had been growing in soil for 18 to 20 weeks at the time they were sacrificed. ND, Not determined.

Line	pHBA Glc Conjugates	
	Total Leaves	Total Stalk
	% DW	
Line 3		
T_0	10.87	1.65
T_1 3-B	18.30	2.44
T_1 3-C	16.15	2.59
Line 4		
T_0	15.22	2.87
T_1 4-A	12.92	2.43
T_1 4-C	14.02	2.37
Line 5		
T_0	13.37	2.52
T_1 5-A	ND	ND

DISCUSSION

Previous attempts to elevate pHBA levels in green plants using the *E. coli ubi*C gene have relied on nuclear transformation for expression of the foreign protein. Using this approach in tobacco, pHBA Glc conjugates accumulated in leaf tissue to a maximum value of 0.52% dry weight, which is at least a 1,000-fold increase over nontransformed control plants (Siebert et al., 1996). Although these results are quite impressive from a metabolic engineering perspective, a commercially viable, plant-based production platform for pHBA will require much higher levels of product accumulation. The potential advantages of using plastid-transformation for CPL-mediated pHBA production in plants are manifold and obvious. First, direct expression of foreign genes in the plastid genome provides an additional safeguard for transgene

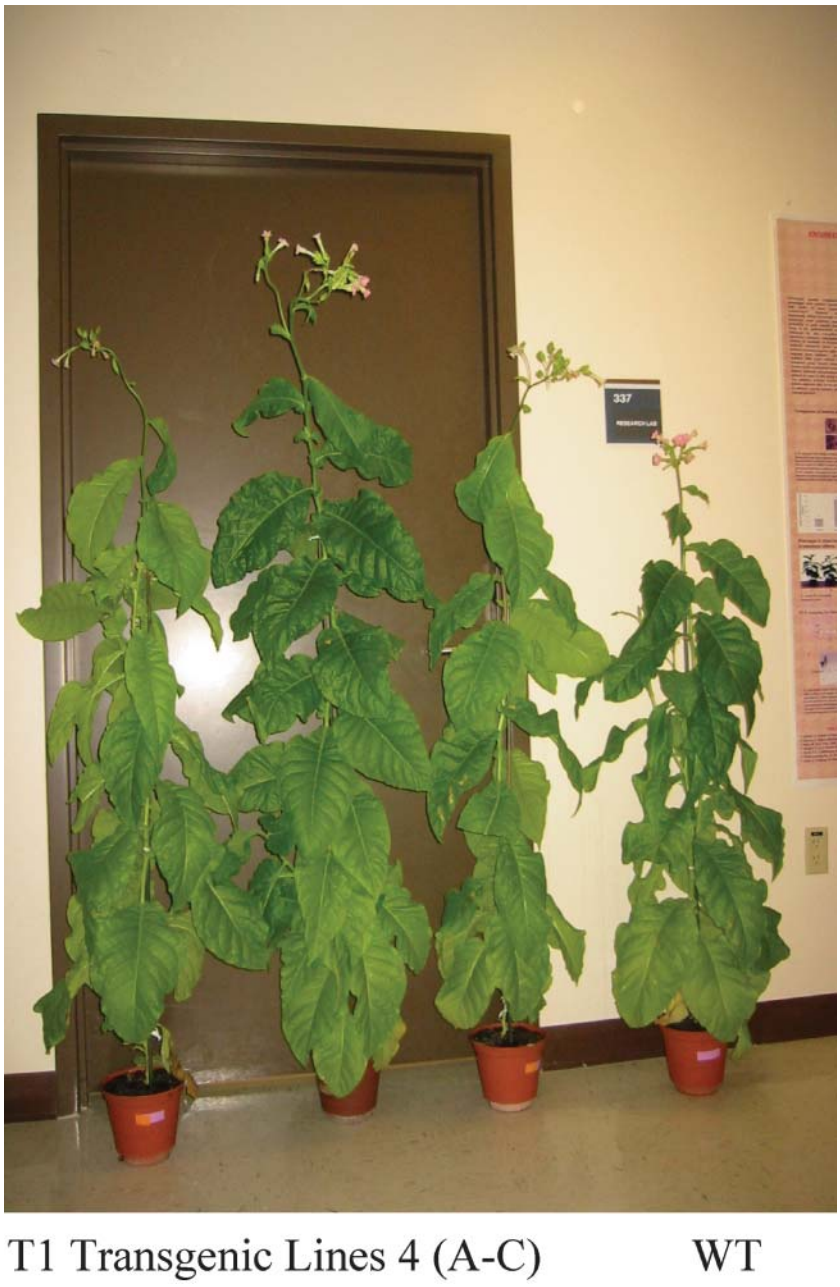

Figure 5. T_1 tobacco plants after 150 d in soil. Shown from left to right are three T_1 plants (lines 4-A, 4-B, and 4-C) and a nontransformed control plant.

containment (Daniell, 2002), since it is widely believed that plastid DNA is maternally inherited in most plant species. Second, CPL's substrate chorimate is largely, if not entirely, synthesized in chloroplasts and other types of plastids (Sommer and Heide, 1998 and refs. therein). Third, using nuclear transformation it is necessary to target CPL to plastids by fusing a chloroplast transit peptide to its N terminus. As already noted, this manipulation is potentially associated with numerous complications that could result in suboptimal levels of CPL enzyme activity in the compartment of interest, thus setting an artificial upper limit to pHBA production in plants that is easily circumvented with plastid transformation. Finally, because of the high copy number of plastid DNA and complete absence of gene silencing mechanisms that are routinely encountered when nuclear transformation is employed (DeCosa et al., 2001; Lee et al., 2003), plastid transformation results in much higher levels of transgene expression than nuclear transformation.

To test the hypothesis that pHBA production in nuclear-transformed plants is currently limited by CPL enzyme activity in the compartment of interest, not chorismate per se, the unmodified *E. coli ubi*C gene was integrated into the tobacco plastid genome. Thus, the foreign protein was directly expressed in the intracellular organelle where its substrate is synthesized. Using this approach we were able to achieve

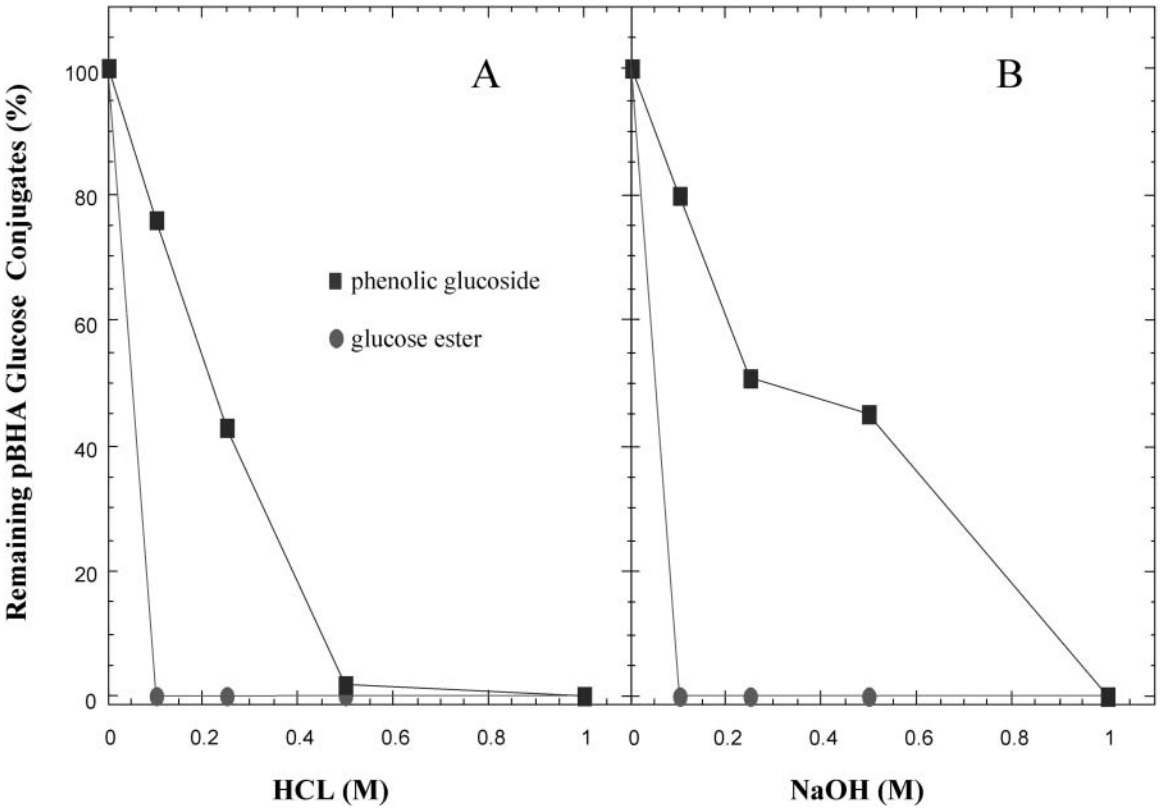

Figure 6. Acid and base hydrolysis of pHBA Glc conjugates. A, Reactions contained an initial concentration of 160 μM pHBA phenolic glucoside (■) or pHBA Glc ester (●) and indicated amounts of HCl (A) or NaOH (B). Following a 48-h incubation period at 60°C, the amount of pHBA released from the Glc conjugates was measured by HPLC.

levels of pHBA Glc conjugates in old leaf tissue that exceeded 25% of the total dry weight when the plants were grown in continuous light (Figs. 3 and 4). This is a 50-fold increase over the maximum value reported for nuclear-transformed tobacco plants expressing the same enzyme (Siebert et al., 1996). More important, the highest CPL enzyme activity that was measured in leaf tissue extracts that were prepared from the plastid-transformed plants was 50,783 pkat/mg of protein; the latter value was obtained from line 3-B, a T_1 plant (see Table I). Based on the molecular mass of CPL (approximately 18,800 D) and the turnover number of the purified recombinant protein determined with the enzyme assay that was used in this study (approximately 2.8 s^{-1}), the CPL content of the leaf tissue extract was approximately 35% of the total soluble protein. In sharp contrast, the highest CPL enzyme activity reported for nuclear-transformed tobacco plants is only 208 pkat/mg of protein (Siebert et al., 1996), which is more than 2 orders of magnitude lower than levels achieved in this study.

It should be emphasized that the CPL-enzyme activity noted above is the average value for total pooled leaf material from a fully mature, plastid-transformed plant that was obtained from two independently prepared cell-free extracts that differed by <5%. The total leaf content of pHBA Glc conjugates in the same plant was 18.3% dry weight (Table I). Given the extraordinarily high levels of CPL enzyme activity that were achieved in this study with plastid transformation, it is quite possible that the latter value represents the upper limit of pHBA production in tobacco leaf at the whole-plant level, although additional studies are necessary to test this hypothesis. Nevertheless, one clear-cut conclusion from our experiments is that CPL is a much better catalyst than HCHL for plant pHBA production when the enzyme is expressed at high levels directly in the plastid compartment.

The substrate for HCHL is pHCA-CoA, a key intermediate in the phenylpropanoid pathway. The highest leaf content of pHBA Glc conjugates reported for HCHL-expressing tobacco was only 2.9% dry weight and the transgenic plants were extremely sick due to a severe depletion of phenylpropanoids (Mayer et al., 2001). As an apparent compensatory mechanism there was a significant increase in transcripts for Phe ammonia-lyase (PAL) and several other enzymes in the phenylpropanoid pathway, including those involved in the synthesis of pHCA-CoA (Mayer et al., 2001). Elevated PAL enzyme activity also accompanied HCHL expression in sugarcane (McQualter et al.,

2004), but the observed 10-fold increase was not sufficient to prevent an almost complete disappearance of leaf chlorogenic acid. Although PAL catalyzes the first step in phenylpropanoid biosynthesis and plays a major role in regulating carbon flow into the pathway (Bate et al., 1994), additional flux control points occur at various downstream branches (Howles et al., 1996). Superimposed on regulation of the individual pathway enzymes is the allocation of chorismate to Phe biosynthesis and partitioning of Phe to PAL; chorismate and Phe are branch point intermediates that are substrates for multiple enzymes. Inability to recruit carbon from the shikimate pathway to replenish Phe and/or downstream phenylpropanoid intermediates in response to rapid consumption of pHCA-CoA could explain HCHL's adverse effects and why this enzyme becomes substrate-limited at much lower levels of pHBA accumulation than CPL.

In striking contrast to the metabolic chaos described above, our plastid-transformed, CPL-expressing plants were healthy and robust and exhibited no discernible negative phenotype despite the fact that more carbon was converted to pHBA. This result is a clear indication that flux through the shikimate pathway was able to keep pace with the massive diversion of chorismate to pHBA and still provide enough carbon for downstream intermediates that are essential for plant growth and development, including pHCA-CoA (HCHL's substrate) and other phenylpropanoids. In bacteria, regulation of carbon flow into the shikimate pathway is largely under transcriptional control and feedback inhibition of the first enzyme in the pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; the aromatic amino acids mediate both processes. Although there is no evidence for a feedback-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in plants, a recent study suggests that reduced thioredoxin plays a role in activation of this enzyme (Entus et al., 2002). Generally speaking, it is believed that all of the plant shikimate pathway enzymes are principally regulated at the transcriptional level and are rapidly induced by wounding, elicitors, and various environmental assaults (Schmid and Amrhein, 1994). Given these observations, it would be very interesting to know if any of the shikimate pathway enzymes and/or phenylpropanoid pathway enzymes were elevated in our transgenic tobacco plants, and if so, to what extent. Indeed, expressing CPL in plastids to create an artificial metabolic sink for chorismate and measuring transcript levels or enzyme activities could provide new insight on the regulation and complex interactions between these two pathways.

The total stalk content of pHBA was at least 5 times lower than the total leaf content in plastid-transformed plants (Table I), and this study provides no explanation for this unexpected observation. Phenylpropanoid biosynthesis in stem tissue is a high-flux pathway that is largely devoted to lignin production, and lignin accounts for approximately 20% of the total dry weight of the stalk. Since all phenylpropanoids are ultimately derived from chorismate, including monolignols, it is reasonable to assume that substrate availability should not be the limiting factor for stalk accumulation of pHBA. The average CPL specific activity in cell-free extracts prepared from total stalk material obtained from fully mature line 3-B was only 8,378 pkat/mg of protein, which is 6-fold lower the corresponding leaf value. However, this result does not necessarily indicate that the process is limited by catalyst. Additional experiments using different promoters and 5′UTRs that might function more effectively in nonphotosynthetic plastids are necessary to see if it is possible to achieve higher stalk levels of pHBA with plastid transformation. In this context, we have recently utilized several regulatory sequences for foreign gene expression in nongreen plastids. For example, the 5′UTR of the T7 gene 10 and 3′UTR of the rps16 gene facilitated 75% transgene expression in nongreen edible parts of carrots containing chromoplasts (grown underground in the dark) and 48% in proplastids, compared to the 100% value in leaf chloroplasts (Kumar et al., 2004a). Similarly, expression of the aphA-6 gene regulated by the T7 gene 10 5′UTR, capable of efficient translation in the dark in proplastids present in nongreen tissues, greatly facilitated stable transformation of the cotton chloroplast genome (Kumar et al., 2004b).

Another challenge for a commercially viable, plant-based production platform is to control the partitioning of pHBA Glc conjugates. As already indicated, the phenolic glucoside and Glc ester are both formed in the cytosol by distinct UDP-glucosyltransferases and are subsequently transported into the vacuole by different carriers. The Glc ester, however, is exquisitely sensitive to acid and base hydrolysis, and this could have a significant impact on the cost of downstream processing in the recovery and purification of polymer-grade pHBA. The ease of hydrolysis of this compound is undoubtedly related to the fact that it is a β-acetal ester. Indeed, it is has been shown that the Glc ester of sinapic acid, a structurally similar compound that is formed in cruciferous plants, has a high free energy of hydrolysis (Mock and Strack, 1993), which allows it to serve as an acyl donor in the enzyme reaction that is catalyzed by sinapoyl-Glc:malate sinapoyltransferase (Strack, 1982). By coexpressing CPL and a UDP-glucosyltransferase that only attaches Glc to the aromatic carboxyl group, it might be possible to partition all or most of the pHBA to the Glc ester.

In summary, pHBA is the major monomer in liquid crystal polymers (LCPs). These thermotropic polyesters have excellent properties, including high strength/stiffness, low melt viscosity, property retention at elevated temperatures, environmental resistance, and low gas permeability (Figuly, 1996). Although LCPs could be used for a variety of new applications, they are currently too expensive for widespread use, largely due to the cost of ingredients. pHBA is also the chemical precursor for parabens

(short-chain alkyl esters of pHBA, generally regarded as safe) that are commonly used as preservatives in food and cosmetics. The Kolbe-Schmitt process (Erickson, 1982) is currently used to synthesize pHBA; this high-temperature, high-pressure carboxylation reaction is relatively expensive. Using plants as a production platform for pHBA via metabolic engineering of the plastid genome is an attractive alternative to petrochemical synthesis, since it is an environmentally sustainable process that is less dependent on nonrenewable resources. Exploiting plants to produce this compound might also lower the cost of manufacture of LCPs and allow them to expand into other niches.

MATERIALS AND METHODS

Construction of the CPL Chloroplast Transformation Vector

The *Escherichia coli ubi*C gene (GenBank accession no. M92628) was amplified from genomic DNA of strain W3110 (Campbell et al., 1978) using two PCR primers. Primer 1 (5′-CTA CTC ATT **gaa ttc aca tgt** CAC ACC CCG CGT TAA C-3′) introduces an *Afl*III site at the CPL start codon and an *Eco*RI site that is immediately upstream from the *Afl*III site. The underlined bases hybridize to the target gene, while the bold lowercase letters indicate the two restriction sites. Primer 2 (5′-CAT CTT ACT **gcg gcc gc**T TTA GTA CAA CGG TGA CGC C-3′) hybridizes at the other end of the gene and introduces a *Not*I site just past the CPL stop codon. The 100-μL PCR reactions contained approximately 100 ng of genomic DNA and both primers at a final concentration of 0.5 μM. The other reaction components were provided by the GeneAmp PCR Reagent kit (Perkin Elmer, Torrance, CA) according to the manufacturer's protocol. Amplification was carried out in a DNA Thermocycler 480 for 22 cycles, each comprising 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; there was a 7-min extension period at 72°C after the last cycle. The PCR product was cut with *Eco*RI and *Not*I, and the resulting fragment was ligated into pBluescript SK (±) (Stratagene, La Jolla, CA) that had been digested with the same enzymes. The ligation reaction mixture was used to transform *E. coli* DH10B electrocompetent cells (GibcoBRL, Gaithersburg, MD) using a BTX Tranfector 100 (Biotechnologies and Experimental Research), and growth was selected on Luria-Bertani media that contained ampicillin (100 μg/mL). Transformants that contained plasmids with a CPL insert were identified by restriction digestion analysis after cleavage with *Eco*RI and *Not*I, and a representative plasmid with no PCR errors was selected for further manipulation. The plasmid containing CPL coding region was digested with *Afl*III and *Not*I and ligated into the plasmid pBSKs+HSA that contains the *psb*A promoter/5′UTR that had been digested with *Nco*I and *Not*I. The resultant plasmid (PpsbA-CPL) was cut with *Eco*RI and *Not*I and cloned into similarly digested pLD-CtV to yield the final construct that was used for chloroplast transformation, pLDK.

Particle Bombardment and Selection of Chloroplast Transformants

Chloroplast transformants were obtained as previously described (Daniell, 1997). Tobacco (*Nicotiana tabacum*) cv Petit Havana leaves were bombarded with the Bio-Rad PDS-1000/He biolistic device. The bombarded leaves were then placed in the dark for 48 h, followed by cutting leaf particles into .5 cm^2 and placed on RMOP media that contained 500 μg/mL spectinomycin for two rounds of selection in petri dishes. Shoots were then cut and transferred to jars that contained MSO media with 500 μg/mL spectinomycin to induce root formation followed by transfer to soil. The transformed and control plants were then placed in a Powers Scientific (Pipersville, PA) diurnal growth chamber with a 16-h-light and 8-h-dark cycle at 26°C ± 1°C. Continuous illumination consisted of 24 h of light. Humidity levels were approximately 55% and average light intensity was approximately 200 $\mu E\ m^{-2}\ s^{-1}$ within the growth chamber.

Confirmation of Site-Specific Integration by PCR

Plant DNA was extracted from transgenic and wild-type tobacco using Qiagen DNeasy plant mini kit (Gaithersburg, MD). The PCR primers, AAAACCCGTCCTCAGTTCGGATTGC (3P) and CCGCGTTGTTTCATCAAGCCTTACG (3M), were used to perform PCR on transgenic and wild-type plant DNA as described previously (Daniell et al., 2004d; Kumar and Daniell, 2004). PCR was carried out in a Perkin Elmer Gene Amp PCR System 2400 under the following conditions: denaturation for 5 min at 94°C, followed by 25 cycles using the following temperature sequence: 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min. After PCR confirmation, the selected shoots were subjected to a second round of selection on RMOP with spectinomycin (500 μg/mL).

Southern-Blot Analysis to Demonstrate Site-Specific Integration and Homoplasmy

These steps were performed essentially as previously described (Daniell et al., 2004d). DNA from T_1 tobacco plants was extracted using Qiagen DNeasy plant mini kit. Plant DNA (2 ug) was digested with *Afl*III and was separated on a 0.8% agarose gel at 50 V for 3 h. The gel was soaked in the depurination solution (0.25 N HCl) for 15 min, and then rinsed twice in water for 5 min. The gel was then soaked in the transfer buffer (0.4 N NaOH, 1 M NaCl) for 20 min and transferred overnight to a nylon membrane. Following transfer, the membrane was rinsed twice with 2× SSC for 5 min, placed on a filter paper, and then cross linked using the GS GeneLinker (Stratagene). The flanking sequence probe (0.81 kb) was obtained by *Bam*HI/*Bgl*II digestion of pUC-ct plasmid, which contains the chloroplast flanking sequences *trnI* and *trnA*. The CPL specific probe (0.3 kb) was obtained by *Sma*I/*Bam*HI digestion of pLDK. Probes were labeled with dCTP ^{32}P using Ready-To-Go DNA labeling beads (-dCTP; Amersham Pharmacia, Piscataway, NJ) and purified using Quant G-50 Micro columns (Amersham Pharmacia). The membrane was then prehybridized with QuikHyb (Stratagene) for 60 min at 68°C, followed by hybridization with radiolabeled probe for 1 h at 68°C. The membrane was then washed twice with 2× SSC in 0.1% SDS at room temperature for 15 min, followed by two washes with 0.1× SSC in 0.1% SDS at 60°C for 15 min. The radiolabeled membrane was then be exposed to Hyperfilm (Amersham Biosciences, Buckinghamshire, UK) and developed in a Konica SRX-101A.

Analysis of pHBA Glc Conjugates

Leaf punches (50–150 mg fresh weight) were used to monitor pHBA levels throughout development. Young, mature, and old tissues were used for this analysis as described in the text and figure legends. Unless otherwise indicated, all steps were conducted at room temperature. The tissue was placed in a Biopulverizer H tube that contained a ceramic bead (QBiogen [Carlsbad, CA]), and 1 mL of 50% (w/v) methanol was added. The tubes were agitated for 40 s using a FastPrep FP120 tissue disrupter (QBiogen) set at 5 m/s, and the samples were then placed on a rotary shaker (400 rpm) for 1 h. Debris was removed by centrifugation and a 50-μL aliquot of the supernatant was taken to dryness in a heated Speed-Vac. The residue was dissolved in 100 μL of 5 mM Tris-HCl, pH 8, for subsequent analysis of pHBA Glc conjugates. Alternatively, the leaf tissue was extracted with 1.0 mL of 5 mM Tris-HCl, pH 8.0, using the same procedure described above. Side-by-side experiments demonstrated that both approaches yield identical results. In some experiments, fully mature tobacco plants were analyzed for whole-leaf and whole-stalk levels of pHBA Glc conjugates. For this type of analysis, all plant material above the ground was harvested, but total leaf and total stalk material were segregated and processed individually. Tissues were lyophilized to dryness and ground to a homogeneous powder in an electrically driven mill. Five to 20 mg of the dry plant material was then extracted with either 1.0 mL of 50% (w/v) methanol or 1.0 mL of 5 mM Tris-HCl, pH 8.0, using the same procedure described above.

pHBA Glc conjugates were analyzed by HPLC using a C_{18} column (Vydac 218TP54 [The Nest Group, Southborough, MA]) that was developed at 1.0 mL/min with a linear gradient (20 min) of 0% to 50% methanol/0.1% formic acid; the separation was performed at room temperature. Elution of the pHBA phenolic glucoside and pHBA Glc ester were monitored at 254 nm. Authentic standards (chemically synthesized and characterized at DuPont) were used to calibrate the HPLC runs, and extinction coefficients for both compounds were accurately determined under the conditions employed.

Peak areas were integrated and values obtained with known amounts of the standards were used to quantitate pHBA Glc conjugates. After accounting for the fraction of the extract that was injected, numbers were corrected to reflect total recovery from the leaf sample analyzed. This, coupled with individual measurements of the dry weight of the plant tissue analyzed (taken from the same leaf, on the same day), enabled the expression of pHBA Glc conjugates as a percentage of total dry weight.

Determination of CPL Enzyme Activity in Leaf and Stalk Cell-Free Extracts

Cell-free extracts were prepared at 0°C to 4°C. The lyophilized leaf and stalk powders described above were the starting materials for this procedure. Approximately 10 mg of the dry powder was transferred to a 1.5-mL polypropylene microfuge tube and 650 μL of a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% β-mercaptoethanol, and 75 mg/mL polyvinylpolypyrrolidone was added. Following a 15-min rehydration period on ice, the sample was hand-homogenized with a small plastic pestle. Debris was removed by centrifugation (14,000*g*, 10 min) and the supernatant was filtered through a 0.22 μm Spin-X Centrifuge Tube Filter (Costar). An aliquot of the filtrate (approximately 200 μL) was then exchanged into buffer Q (50 mM Tris-HCl, pH 7.3, 0.3 M NaCl, 5 mM $MgCl_2$, 6% [w/v] glycerol, 5 mM dithiothreitol) using the following procedure. The sample was concentrated approximately 10-fold using a Microcon YM-10 concentrator (Millipore, Bedford, MA) and the retentate was diluted with 200 μL of buffer Q. This step was repeated three times to yield the final preparations that were used to measure CPL enzyme activity.

CPL enzyme activity was measured spectrophotometrically using a continuous assay that is based on the increase in A_{246} that occurs when chorismate is converted to pHBA. Reactions were carried out at 37°C in a quartz cuvette that contained 90 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 100 μM purified barium chorismate (Siebert et al., 1994). Assays were initiated with various amounts of cell-free extract. Initial rates of product formation were used to calculate CPL specific activities (expressed as picokatals per milligram of protein). An empirically derived molar extinction coefficient of 11,220 M^{-1} for pHBA at 246 nm (determined under the same conditions) was used for these calculations. Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

Received July 16, 2004; returned for revision October 15, 2004; accepted October 17, 2004.

LITERATURE CITED

Bartholomew DM, Van Dyk DE, Lau S-ZC, O'Keefe DP, Rea PA, Viitanen PV (2002) Alternate energy-dependent pathways for the vacuolar uptake of glucose and glutathione conjugates. Plant Physiol **30:** 1562–1572

Bate NJ, Orr J, Ni W, Meromi A, Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ, Elkind Y (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc Natl Acad Sci USA **91:** 7608–7612

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Campbell JL, Richardson CC, Studier FW (1978) Genetic recombination and complementation between bacteriophage T7 and cloned fragments of T7 DNA. Proc Natl Acad Sci USA **75:** 2276–2280

Daniell D, Carmona-Sanchez O, Burns BB (2004b) Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. *In* R Fischer, S Schillberg, eds, Molecular Farming Weinheim. WILEY-VCH, Verlag, pp 113–133

Daniell H (1997) Transformation and foreign gene expression in plants mediated by microprojectile bombardment. Methods Mol Biol **62:** 463–489

Daniell H (2002) Molecular strategies for gene containment in transgenic crops. Nat Biotechnol **20:** 581–586

Daniell H (2004) Medical molecular pharming: therapeutic recombinant antibodies, biopharmaceuticals, and edible vaccines in transgenic plants engineered via the chloroplast genome. *In* RM Goodman, ed, Encyclopedia of Plant and Crop Science. Marcel Decker, New York, pp 704–710

Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2004c) Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine (in press)

Daniell H, Cohill P, Kumar S, Dufourmantel N, Dubald M (2004a) Chloroplast genetic engineering. *In* H Daniell, C Chase, eds, Molecular Biology and Biotechnology of Plant Organelles, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 423–468

Daniell H, Datta R, Varma S, Gray S, Lee SB (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat Biotechnol **16:** 345–348

Daniell H, Dhingra A (2002) Multigene engineering: Dawn of an exciting new era in biotechnology. Curr Opin Biotechnol **13:** 136–141

Daniell H, Kahn M, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci **7:** 84–91

Daniell H, Lee SB, Panchal T, Wiebe PO (2001a) Expression of cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol **311:** 1001–1009

Daniell H, Muthukumar B, Lee SB (2001b) Marker free transgenic plants: the chloroplast genome without the use of antibiotic selection. Curr Genet **39:** 109–116

Daniell H, Ruiz ON, Dhingra A (2004d) Chloroplast genetic engineering to improve argonomic traits. Methods Mol Biol **286:** 111–137

DeCosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the *Bt Cry2Aa2* operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol **19:** 71–74

DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol **127:** 852–862

Devine AL, Daniell H (2004) Chloroplast genetic engineering for enhanced agronomic traits and expression of proteins for medical/industrial applications. *In* SG Møller, ed, Plastids, Vol 13. Blackwell Publishing, Oxford, pp 283–323

Dhingra A, Portis AR, Daniell H (2004) Enhanced translation of a chloroplast-expressed *Rbc*S gene restores small subunit levels and photosynthesis in nuclear antisense *Rbc*S plants. Proc Natl Acad Sci USA **101:** 6315–6320

Dufourmantel N, Pelissier B, Garçon F, Peltier JM, Tissot G (2004) Generation of fertile transplastomic soybean. Plant Mol Biol (in press)

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop HU (1999) *In vivo* analysis of plastid *psb*A, *rbc*L and *rpl*32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J **19:** 333–345

Entus R, Poling M, Hermann KM (2002) Redox regulation of arabidopsis 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. Plant Physiol **129:** 1866–1871

Erickson B (1982) Salicylic acid and related compounds. *In* M Grayson, ed, Kirk-Othmer Encyclopedia of Chemical Technology. Wiley, New York, Vol 20, pp 517–519

Fernandez-San Millan A, Mingeo-Castel AM, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol J **1:** 71–79

Figuly GD (1996) Liquid crystalline polymers (thermotropic polyesters) in polymeric materials., *In* JC Salamone, ed, Encyclopedia, Vol 5. CRC Press, Boca Raton, FL, pp 3731–3740

Gasson MJ, Kitamura Y, McLauchlan WR, Narbad A, Parr AJ, Parsons EL, Payne J, Rhodes MJ, Walton NJ (1998) Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J Biol Chem **273:** 4163–4170

Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. Nucleic Acids Res **19:** 4083–4089

Guda C, Lee SB, Daniell H (2000) Stable expression of biodegradable protein based polymer in tobacco chloroplasts. Plant Cell Rep **19:** 257–262

Herrmann KM, Weaver LM (1999) The shikimate pathway. Annu Rev Plant Physiol Plant Mol Biol **50:** 473–503

Howles PA, Sewalt VJW, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon RA

(1996) Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol **112:** 1617–1624

Hrazdina G, Jensen RA (1992) Spatial organization of enzymes in plant metabolic pathways. Annu Rev Plant Physiol **43:** 241–267

Kota M, Daniell H, Varma S, Garczynski SF, Gould F, William MJ (1999) Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci USA **96:** 1840–1845

Kumar S, Daniell H (2004) Engineering the chloroplast genome for hyper-expression of human therapeutic proteins and vaccine antigens in recombinant protein protocols. Methods Mol Biol **267:** 365–383

Kumar S, Dhingra A, Daniell H (2004a) Plastid-expressed *betaine aldehyde dehydrogenase* gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol **136:** 2843–2854

Kumar S, Dhingra A, Daniell H (2004b) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol (in press)

Lee SB, Kwon HB, Kwon SJ, Park SC, Jeong MJ, Han SE, Byun MO, Daniell H (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. Mol Breed **11:** 1–13

Leelavathi S, Gupta N, Maiti S, Ghosh A, Reddy VS (2003) Overproduction of an alkali- and thermo-stable xylanase in tobacco chloroplasts and efficient recovery of the enzyme. Mol Breed **11:** 59–67

Leelavathi S, Reddy VS (2003) Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. Mol Breed **11:** 49–58

Li Y, Baldauf S, Lim E-K, Bowles DJ (2001) Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana.* J Biol Chem **276:** 4338–4343

Lim EK, Doucet CJ, Li Y, Elias L, Worrall D, Spencer SP, Ross J, Bowles DJ (2002) The activity of Arabidopsis glycosyltransferases toward salicylic acid, 4-hydroxybenzoic acid, and other benzoates. J Biol Chem **277:** 586–592

Loscher R, Heide L (1994) Biosynthesis of p-hydroxybenzoate from p-coumarate and p-coumaroyl-coenzyme A in cell-free extracts of *Lithospermum erythrorhizon* cell cultures. Plant Physiol **106:** 271–279

Lossl A, Eibl C, Harloff HJ, Jung C, Koop HU (2003) Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. Plant Cell Rep **21:** 891–899

Mayer MJ, Narbad A, Parr AJ, Parker ML, Walton NJ, Mellon FA, Michael AJ (2001) Rerouting the plant phenylpropanoid pathway by expression of a novel bacterial enoyl-CoA hydratase/lyase enzyme function. Plant Cell **13:** 1669–1682

McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology (N Y) **13:** 362–365

McQualter RB, Chong BF, Meyer K, Van Dyk DE, O'Shea MG, Walton NJ, Viitanen PV, Brumbley SM (2004) Initial evaluation of sugarcane as a production platform for p-hydroxybenzoic acid. Plant Biotechnol J (in press)

Mitra A, Kitamura Y, Gasson MJ, Narbad A, Parr AJ, Payne J, Rhodes MJC, Sewter C, Walton NJ (1999) 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL): an enzyme of phenylproanoid chain cleavage from *Pseudomonas.* Arch Biochem Biophys **365:** 10–16

Mock HP, Strack D (1993) Energetics of the uridine 5′-disphosphoglucose: hydroxycinnamic acid acyl-glucosyltransferase reaction. Phytochemistry **32:** 575–579

Molina A, Herva-Stubbs S, Daniell H, Mingo-Castel AM, Veramendi J (2004) High yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnol **2:** 141–153

Robinson C, Ellis RJ (1984) Transport of proteins into chloroplasts. The precursor of small subunit of ribulose bisphosphate carboxylase is processed to the mature size in two steps. Eur J Biochem **142:** 342–346

Robinson C, Ellis RJ (1985) The effect of incorporation of amino acid analogues on the import and processing of chloroplast polypeptides. Eur J Biochem **152:** 67–73

Ross J, Li Y, Lim E-K, Bowles DJ (2002) Higher plant glycosyltransferases. Genome Biol **2:** REVIEWS3004

Ruiz ON, Hussein H, Terry N, Daniell H (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol **132:** 1344–1352

Schmid J, Amrhein N (1995) Molecular organization of the shikimate pathway in higher plants. Phytochemistry **39:** 737–749

Schnitzler J-P, Madlung J, Rose A, Seitz HU (1992) Biosynthesis of p-hydroxybenzoic acid in elicitor-treated carrot cell cultures. Planta **188:** 594–600

Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J **4:** 25–32

Siebert M, Severin K, Heide L (1994) Formation of 4-hydroxybenzoate in Escherichia coli: characterization of the *ubi*C gene and its encoded enzyme chorismate pyruvate-lyase. Microbiol **140:** 897–904

Siebert M, Sommer S, Li SM, Wang ZX, Severin K, Heide L (1996) Genetic engineering of plant secondary metabolism: accumulation of 4-hydroxybenzoate glucosides as a result of the expression of the bacterial *ubi*C gene in tobacco. Plant Physiol **112:** 811–819

Sommer S, Heide L (1998) Expression of bacterial chorismate pyruvate-lyase in tobacco: evidence for the presence of chorismate in the plant cytosol. Plant Cell Physiol **39:** 1240–1244

Sommer S, Kohle A, Kazufumi Y, Shimomura K, Bechthold A, Heide L (1999) Genetic engineering of shikonin biosynthesis hairy root cultures of *Lithospermum erythrorhizon* transformed with the bacterial *ubi*C gene. Plant Mol Biol **39:** 683–693

Sommer S, Siebert M, Bechtold A, Heide L (1998) Specific induction of secondary product formation in transgenic plant cell cultures using an inducible promoter. Plant Cell Rep **17:** 891–896

Staub JM, Garcia B, Graves J, Hajdukiewicz PTJ, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, et al (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat Biotechnol **18:** 333–338

Stern DB, Gruissem W (1987) Control of plastid gene expression: 3′ inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. Cell **51:** 1145–1157

Strack D (1982) Development of 1-O-sinapoyl-β-D-glucose:L-malate sinapoyltransferase activity in cotyledons of red radish (*Raphanus stivus* L. var. *stivus*). Planta **155:** 31–36

Van den Broeck G, Timko MP, Kausch AP, Cashmore AR, Montagu MV, Herrera-Estrella L (1985) Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose-1,5-bisphosphate carboxylase. Nature **313:** 358–362

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet **17:** 449–453

Wasmann CC, Reiss B, Bohnert HJ (1988) Complete processing of a small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from pea requires the amino acid sequenc Ile-Thr-Ser. J Biol Chem **263:** 617–619

Watson J, Koya V, Leppla SH, Daniell H (2004) Expression of *Bacillus anthracis* protective antigen in transgenic tobacco chloroplasts: development of an improved anthrax vaccine in a non-food/feed crop. Vaccine **22:** 4374–4384

Yazaki K, Heide L, Tabata M (1991) Formation of p-hydroxybenzoic acid from p-coumaric acid and by cell free extract of *Lithospermum erythrorhizon* cell cultures. Phytochemistry **30:** 2233–2236