Expression of Bacterial Chorismate Pyruvate-Lyase in Tobacco: Evidence for the Presence of Chorismate in the Plant Cytosol

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The bacterial gene ubiC encodes chorismate pyruvate-lyase, an enzyme which converts chorismate to 4-hydroxybenzoate and which is not normally present in plants. The UbiC protein was expressed in tobacco, with targeting of the gene product either to the plastids or to the cytosol. In both cases, chorismate pyruvate-lyase activity and a resulting formation of 4-hydroxybenzoate was detected. This suggests that chorismate, a metabolite of the shikimate pathway, is present not only in the plastids but also in the cytosol of plant cells.

Key words: Chorismate — 4-Hydroxybenzoic acid — Nicotiana tabacum — ubiC.

Genetic engineering of secondary metabolism in plants is a newly emerging field of research, which may open routes for the improved production of pharmaceuticals (Yun et al. 1992) or the development of crops with improved disease resistance (Hain et al. 1993). In contrast to the genetic engineering of bacteria, consideration of the intracellular compartmentation and transport of proteins and metabolites is of essential importance in the genetic modification of plant metabolism. We have recently expressed the bacterial ubiC gene, encoding chorismate pyruvate-lyase (CPL), in tobacco plants, using a transit peptide for plastidic targeting of the gene product (Siebert et al. 1996). CPL converts chorismate to 4-hydroxybenzoate (4HB) and is not normally present in plants. The expression of this ubiC construct in tobacco resulted in a formation of 4HB as a new, artificial secondary metabolite in tobacco, which was accumulated in form of glucosides (Siebert et al. 1996) and also incorporated into the cell wall (Li et al. 1997). First experiments suggested that this transformation may increase plant disease resistance (Heide et al. 1997). Using an inducible plant promoter, the expression of the ubiC gene could be closely regulated by the addition of an inducer substance (Sommer et al. 1998).

In the present study, we attempted to express the ubiC gene in tobacco with cytosolic rather than with plastidic targeting of its gene product, CPL. Chorismate, the substrate of this enzyme, is a metabolite of the shikimate pathway. Whether this pathway is exclusively localized in plastids, or whether a parallel shikimate pathway exists in the cytosol, is discussed controversially (Hrazdina and Jensen 1992, Forlani et al. 1994, Schmid and Amrhein 1995). So far, all published gene sequences for shikimate pathway enzymes in plants contain transit peptide sequences for plastidic import (Schmid and Amrhein 1995). However, a gene apparently encoding a cytosolic isoenzyme of chorismate mutase has recently been described (Eberhard et al. 1996). Expression of ubiC with cytosolic targeting of its gene product, CPL, in tobacco and detection of a resulting formation of 4HB should provide an elegant method to investigate in vivo whether chorismate is present in the cytosol.

Experimental procedures—Escherichia coli XL1-Blue is described by Bullock et al. (1987), *Agrobacterium tumefaciens* strain LBA4404 by Hoekema et al. (1983), *E. coli* AN92 by Young et al. (1971), and *Nicotiana tabacum* cv. Wisconsin 38 TET (*N. tabacum* W38 TET) by Gatz et al. (1991). Cell cultures of the tobacco plants were established by cultivating pieces of sterile grown seedlings on MS medium (Murashige and Skoog 1962), containing per liter: 4.46 g of MS salts (Sigma), 30 g of sucrose, 0.05 mg of kinetin, 0.5 mg of 2,4-D and 10 g of Bacto Agar (Difco, Detroit, MI); pH 5.7. After 4 to 6 weeks the generated callus tissues were transfected into 300 ml conical flasks; each flask contained 75 ml of liquid MS medium, containing per liter: 4.46 g of MS salts, 30 g of sucrose, 1 mg of NAA, and 0.2 mg of 6-benzylaminopurine; pH 5.7. The cultures were maintained in the dark at 25°C and 80 rpm, with subculturing at 14-d intervals. For transformants of *N. tabacum* W38 TET all media contained both kanamycin (100 mg liter⁻¹) and hygromycin (15 mg liter⁻¹). For transformants of *N. tabacum* cv. Petit Havana SR1 all media contained kanamycin (100 mg liter⁻¹). The plasmid pTOP is registered in the EMBL database under the accession number Z37515 and described by Gatz et al. (1992). pROK1 (Baulcombe et al. 1986) is here named p3SS, and pLMU3 is described by Siebert et al. (1992), p3SS-TP-UbiC by Siebert et al. (1996), and pTOP-TP-UbiC and pTOP-TP21-UbiC by Sommer et al. (1998). To construct pUbiC(G), a PCR frag-
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The expression of UbiC was obtained from the plasmid pALMU3 with the primers (+) 5'-dGGA GAG TCT CCG TG and (-) 5'-dATT CTG CTG CAG ACT CCA CCA CTC-3' and was ligated into the Smal site of pUC18 to give plasmid pUbiC(G). To construct pUbiCv, a PCR fragment was obtained from the plasmid pALMU3 with the primers (+) 5'-dA GAT CTA GAC AAT ATG GCA CAC CCC GGC TT-3' and (-) 5'-dGAA GAT CTA TTC TGC GTC AGA CTC C-3' and was ligated into the Smal site of pUC18 to give plasmid pUbiCv. The plasmid pUbiCv was digested with BglII, and the resulting fragment, containing the ubiCv gene, was ligated into the BamHI site of p35S to obtain p35S-UbiCv. These constructs were transformed into E. coli XL1-Blue, and the correct orientation of the insert was confirmed by restriction analysis. To construct pTOP-UbiCv, the plasmid pUbiCv was digested with SalI and KpnI and the fragment containing ubiCv was ligated into pTOP, which has been restricted with the same enzymes. Constructs were transformed into E. coli XL1-Blue. Transformation of Agrobacterium tumefaciens strain LBA4404 was carried out as described by Höfgen and Willmitzer (1988). Leaf disc transformation and regeneration of N. tabacum Petit Havana SR1 and N. tabacum W38 TET was performed according to Baumann et al. (1987). N. tabacum W38 TET was already transformed with pTET and therefore showed a kanamycin resistance and a constitutive expression of the repressor protein (Gatz et al. 1991). For induction experiments, chlorotetacycline was dissolved in water (2 mg ml⁻¹) and added to cell cultures three days after inoculation to the final concentrations of 2 mg liter⁻¹ medium (Sommer et al. 1998). For determination of CPL activities in bacteria, the corresponding plasmids were transformed into E. coli AN 92. Bacterial enzyme extracts were prepared and CPL activities were determined as described by Siebert et al. (1992). For the determination of CPL activity and the measurement of 4HB derivatives in plant cell cultures, the methods described by Siebert et al. (1996) were used.

Expression of UbiC in the plant cytosol—Targeting to the cytosol is the default mechanism for the expression of nuclear-encoded, soluble plant proteins. Therefore, in early experiments on the expression of the UbiC protein in the cytosol, the bacterial gene was directly fused to the 35S plant promoter (Siebert 1994). After transformation of this 35S-UbiC construct into tobacco, however, no significant CPL activity and 4HB accumulation was detected, in contrast to tobacco transformed with a fusion of the ubiC gene to a sequence of a transit peptide for plastidic import (35S-TP-UbiC construct; Siebert et al. 1996). A possible reason for this failure of 35S-UbiC expression was seen in the nucleotide sequence surrounding the translation start of the bacterial ubiC gene, which differs from the consensus sequence for plant translational starts (Fig. 1). Especially the thymidine nucleotide in position +4 of the structural gene is very rare in plants, occurring in only 2% of examined plant genes (Lütcke et al. 1987). In order to ensure equal translation efficiency of the differentially targeted ubiC gene constructs we therefore decided to change the translation start of ubiC, making it exactly identical to the translational start sequence of the constructs with plastidic targeting (Fig. 1). This modification results, besides changes in the 5'-untranslated region, in a Ser → Ala exchange in the second amino acid of the ubiC gene product. The effect of this amino acid exchange on the CPL activity was examined by construction of vector pUbiC(G). This vector differed from the previously described vector pUbiC (Siebert et al. 1996) only by the T → G exchange in position +4 of the structural gene. Upon expression of both vectors in E. coli AN 92, identical CPL activities were obtained (267 and 265 pkat (mg protein)⁻¹, respectively), showing that the modification did not impair enzyme activity.

Subsequently, the ubiCv gene with the modified translation start shown in Figure 1 was constructed by PCR from pALMU3 and ligated into pUC18 to give pUbiCv. Sequencing of the entire insert confirmed that no mutations had occurred during the PCR amplification. ubiCv was ligated into the binary vector p35S, placing it under control of the constitutive 35S plant promoter. The binary vector construct was used for Agrobacterium tumefaciens-mediated transformation of leaf discs of N. tabacum cv. Petit Havana SR1. 20 plants representing different transformation events were regenerated under a selection pressure for antibiotic resistance, and plant cell cultures were established from these lines.

Constitutive expression of ubiCv—The presence of the ubiC gene in the genomic DNA of the transgenic lines was shown by the Southern hybridization technique described in our previous study (Sommer et al. 1998). Transgenic plant seeds obtained by self-pollination were germinated in

Fig. 1 Translation start sequences of the ubiC gene constructs, compared to published consensus sequences of plants. 35S-UbiC: 35S plant promoter + bacterial ubiC gene. 35S-TP-UbiC: 35S plant promoter + transit peptide for plastidic import + bacterial ubiC gene. 35S-UbiCv: 35S plant promoter + bacterial ubiC gene with modified translation start. Top-UbiCv: inducible plant promoter + bacterial ubiC gene with modified translation start.
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The presence of kanamycin, and a 1:3 distribution (ranging from 1:2.6 to 1:3.7) between antibiotic-sensitive and antibiotic-resistant phenotypes was observed. Therefore, the antibiotic resistance gene had been stably integrated into the genome at one locus. Cell suspension cultures established from the transgenic lines were used for the following investigations, since they show less variation than intact plant material. Northern blot analysis of five randomly selected transgenic lines clearly showed the UbiC transcripts at the expected size of 0.8 kb (Fig. 2). As in our previous studies (Siebert et al. 1996, Sommer et al. 1998), no UbiC mRNA was detected in the untransformed control. Likewise, CPL activity was detected in the transgenic lines but not in the untransformed control (Table 1). The enzyme activity measured in the different lines approximately correlated with the UbiC transcript level estimated from the Northern blots.

**Accumulation of 4HB derivatives**—The gene product of UbiC, chorismate pyruvate-lyase, converts chorismate into 4HB. Untransformed control cultures contained only minimal amounts of this compound. Expression of UbiC in transgenic plants led to a clearly detectable accumulation of 4HB derivatives (Table 1), with 4HB O-β-D-glucoside as the main product. This product has been isolated in preparative amounts and identified by ‘H NMR and 13C NMR in our previous study (Siebert et al. 1996). It was now identified in comparison to the authentic reference substance by HPLC and UV spectroscopy. The compound was also hydrolyzed with hydrochloric acid to yield free 4HB, which was again identified by HPLC and UV spectroscopy.

The amount of 4HB derivatives in the 35S-UbiC lines (with cytosolic targeting of CPL) was not very different of that found previously in a transgenic line with comparable CPL activity and plastidic targeting (line Tim III, Sommer et al. 1998).

### Table 1  Chorismate pyruvate-lyase activity and accumulation of 4-hydroxybenzoate derivatives in cell culture lines expressing different UbiC gene constructs

<table>
<thead>
<tr>
<th>Line</th>
<th>CPL activity (pkt/mg protein)</th>
<th>Content of 4HB derivatives (μmol/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR1 (control)</td>
<td>&lt;0.5</td>
<td>&lt;0.75</td>
</tr>
<tr>
<td>35S-UbiC lines:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 7</td>
<td>1.5</td>
<td>0.85</td>
</tr>
<tr>
<td>Line 8</td>
<td>2.7</td>
<td>2.26</td>
</tr>
<tr>
<td>Line 9</td>
<td>1.6</td>
<td>1.14</td>
</tr>
<tr>
<td>Line 12</td>
<td>2.7</td>
<td>1.31</td>
</tr>
<tr>
<td>Line 13</td>
<td>2.0</td>
<td>1.76</td>
</tr>
<tr>
<td>Tim line III*</td>
<td>2.3*</td>
<td>3.40*</td>
</tr>
<tr>
<td>35S-TP-UbiC lines:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 1</td>
<td>26.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Line II</td>
<td>19.8</td>
<td>19.2</td>
</tr>
</tbody>
</table>

* Tim III is a transgenic line with plastidic targeting of the UbiC gene product; data are from Sommer et al. (1998).

* Measured after hydrolysis with 2 M hydrochloric acid to yield free 4HB. All data are mean values of at least two independent determinations.
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et al. 1998; this line differed from the 3SS-TP-UbiC lines by inclusion of 21 amino acids of the rubisco small subunit between the transit peptide and the ubiC gene product). The most active 3SS-TP-UbiC lines, however, showed over ten times more CPL activity and 4HB accumulation than the 3SS-UbiCv lines (Table 1).

Inducible expression of ubiCv—The 3SS-UbiCv and the 3SS-TP-UbiCv constructs were under control of the same promoter and contained identical translation start sequences (Fig. 1). They may therefore be expected to be expressed with similar transcription and translation efficiency. Nevertheless, expression of the 3SS-UbiCv construct (with cytosolic localization of the gene product) yielded considerably lower enzyme activities than expression of the 3SS-TP-UbiCv construct.

We speculated that high expression of UbiC in the cytosol may have detrimental effects on the transformed plant cell, e.g. due to toxicity of the phenolic product 4HB, or due to the diversion of chorismate from its normal metabolic flow. If so, transformation events leading to a high expression of CPL in the cytosol may not lead to the regeneration of viable transgenic plants. To avoid such a negative selection, we decided to attempt an expression of the ubiCv construct under control of the tetracycline-inducible TOP promoter (Gatz et al. 1992), which is tightly repressed during the regeneration of transgenic plants and which can be induced at any desired time point by the addition of chlorotetracycline.

The ubiCv sequence was therefore ligated into the binary vector pTOP, placing it under control of this inducible plant promoter. The resulting pTOP-UbiCv construct was used for Agrobacterium tumefaciens-mediated transformation of leaf disks of Nicotiana tabacum Wisconsin 38 TET. Ten plants representing different transformation events were regenerated under a selection pressure for antibiotic resistance, and cell cultures of these lines were established. The presence of the ubiC gene in the genomic DNA was again shown by Southern blotting (data not shown). Germination of transgenic plant seeds in the presence of kanamycin and hygromycin gave a 1:3 distribution between antibiotic-sensitive and antibiotic-resistant phenotypes, showing that the antibiotic resistance gene had been stably integrated into the genome at one locus.

Upon addition of chlorotetracycline as inducer to the cell cultures, the appearance of ubiC mRNA and CPL activity could be shown, similar to our previous study with the TOP-TP-UbiCv construct (Sommer et al. 1998). However, CPL activity of the induced TOP-UBiCv lines (average 1.8 pkat (mg protein)^{-1}) was not higher than that of the constitutively expressing 3SS-UbiCv lines (average 2.0 pkat (mg protein)^{-1}) and did not reach the activity levels of the previously examined, induced TOP-TP-UbiCv lines with plastidic targeting (approximately 20 pkat (mg protein)^{-1}; Sommer et al. 1998).

When ubiC expression was induced in the TOP-UBiCv lines, no detrimental effect on the cell viability was observed. From these data, it appears unlikely that negative selection against high expression of CPL in the cytosol is the reason for the failure to obtain ubiCv transformants with high activity.

Conclusions—in plants, plastids are the primary locus of the shikimate pathway. We have now transformed tobacco with the bacterial gene ubiC, after modification of the translation start for the expression in plants. The gene was integrated into the nuclear genome using Agrobacterium tumefaciens. Translation will therefore occur at the cytosolic ribosomes. The gene product, chorismate pyruvate-lyase, is expected to remain exclusively in the cytosol, since it does not recognize sequences for the highly specific import processes into plastids, mitochondria, or the golgi apparatus (Bar-Peled et al. 1996). Expression of this construct resulted both in active CPL and in a formation of 4-hydroxybenzoate, the product of the CPL reaction. This finding suggests that chorismate was present in the cytosol and served as substrate for the CPL reaction.

The existence of a cytosolic isoform of chorismate mutase has recently been confirmed by molecular biological data (Eberhard et al. 1996). Our results indicate that this enzyme may in fact catalyze a formation of prephenate in the plant cytosol. The physiological significance of this pathway is unknown at present, and it is not clear whether chorismate is synthesized in the cytosol or simply exported from the plastids.

The present study demonstrates that in the genetic engineering of plants the intracellular targeting of foreign gene constructs may have a strong influence on the resulting expression levels. Plastidic targeting of the ubiC gene product yielded highest enzyme activities and 4HB accumulation, in spite of the fact that in this case the translation product has to be transported through the chloroplast envelope and processed by a stromal protease in order to liberate the fully active enzyme protein. The correct transport and processing of the TP-UbiC fusion protein has been demonstrated previously (Siebert et al. 1996). Expression of ubiC without a transit peptide sequence, resulting in a cytosolic localization of the gene product, yielded considerably lower enzyme activities, even when the same promoter and identical translation start sequences were used. Negative selection against high expression of CPL in the cytosol could be ruled out by experiments with an inducible promoter. The comparatively low CPL activity obtained with ubiC constructs without transit peptide may therefore be attributed to a more rapid turnover of the mRNA of these constructs, and/or to a more rapid degradation of the protein, e.g. by the action of cytosolic proteases.
We thank F. Maier for excellent technical assistance with the cell cultures, L. Westrich for sequencing and A. Bechthold and A. Wagner for helpful discussions. This work was supported by the Dr. Hilmer-Foundation (to S.S.) and by the DFG (to L.H.).

References


(Received May 11, 1998; Accepted July 31, 1998)