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Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance

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Abstract. Certain higher plants synthesize and accumulate glycine betaine, a compound with osmoprotectant properties. Biosynthesis of glycine betaine proceeds via the pathway choline → betaine aldehyde → glycine betaine. Plants such as tobacco (*Nicotiana tabacum* L.) which do not accumulate glycine betaine lack the enzymes catalyzing both reactions. As a step towards engineering glycine betaine accumulation into a non-accumulator, spinach and sugar beet complementary-DNA sequences encoding the second enzyme of glycine-betaine synthesis (betaine aldehyde dehydrogenase, BADH, EC 1.2.1.8) were expressed in tobacco. Despite the absence of a typical transit peptide, BADH was targeted to the chloroplast in leaves of transgenic plants. Levels of extractable BADH were comparable to those in spinach and sugar beet, and the molecular weight, isoenzyme profile and K_m for betaine aldehyde of the BADH enzymes from transgenic plants were the same as for native spinach or sugar beet BADH. Transgenic plants converted supplied betaine aldehyde to glycine betaine at high rates, demonstrating that they were able to transport betaine aldehyde across both the plasma membrane and the chloroplast envelope. The glycine betaine produced in this way was not further metabolized and reached concentrations similar to those in plants which accumulate glycine betaine naturally. Betaine aldehyde was toxic to non-transformed tobacco tissues whereas transgenic tissues were resistant due to detoxification of betaine aldehyde to glycine betaine. Betaine aldehyded ehydrogenase is therefore of interest as a potential selectable marker, as well as in the metabolic engineering of osmoprotectant biosynthesis.

Key words: *Nicotiana* (transgenic) – Betaine aldehyde dehydrogenase – Chloroplast targeting – Glycine betaine – Selectable marker

Abbreviations: BADH = betaine aldehyde dehydrogenase; bp = base pairs; FAB-MS = fast atom bombardment-mass spectrometry; GAPDH = NADP-linked glyceraldehyde-3-phosphate dehydrogenase

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Introduction

In higher plants and other organisms, a common metabolic adaptation to salinity or drought stress is the accumulation of osmoprotectants. Osmoprotectants are small molecules that can benefit osmotically stressed cells in two ways: by acting as nontoxic cytoplasmic osmolytes to raise osmotic pressure, and by stabilizing enzymes and membranes against damage by high salt levels (Wyn Jones 1984). Osmoprotectants fall into two chemical classes: polyols and their derivatives (Tarczynski et al. 1993), and small zwitterions such as amino acids and betaines (Somero 1986; Csonka and Hanson 1991). Glycine betaine is among the most effective of these and occurs in several families of higher plants, particularly in species adapted to dry and saline environments (e.g. Chenopodiaceae, Poaceae) (Rhodes and Hanson 1993). However, many higher plants do not accumulate glycine betaine or any other osmoprotectant, and this has led to interest in the metabolic engineering of the glycine betaine biosynthesis pathway as an approach for enhancing stress resistance (LeRudulier et al. 1984; McCue and Hanson 1990).

Higher plants synthesize glycine betaine in chloroplasts via the pathway: choline → betaine aldehyde → glycine betaine (Rhodes and Hanson 1993). The first step is catalyzed by choline monooxygenase (Brouquisse et al. 1989), the second by betaine aldehyde dehydrogenase (BADH) (Weigel et al. 1986). In spinach, BADH is a homodimer of nuclear-encoded subunits with a molecular weight of about 60 kDa (Arakawa et al. 1987; Weretilnyk and Hanson 1988; Weretilnyk and Hanson 1989). Spinach BADH is localized mainly (90%) in the chloroplast stroma (Weigel et al. 1986). Complementary-DNA (cDNA) clones encoding BADH from spinach (Weretilnyk and Hanson 1990) and sugar beet (henceforth, beet) (McCue and Hanson 1992) have been isolated. Although these clones presumably code for stromal enzymes, they lack typical transit peptide sequences. Comparison of deduced and determined amino-acid sequences indicates that their transit peptides contain at most eight residues,

Fig. 1. N-terminal amino-acid sequences deduced from spinach and beet BADH cDNAs (Weretilnyk and Hanson 1990; McCue and Hanson 1992). Residues after the deduced translational start site are in *bold* type. *Asterisks* denote stop codons. The *arrow* denotes a trypsin cleavage site. The *underlined* sequence is from a tryptic peptide obtained from purified spinach BADH

and might be absent altogether (Fig. 1). Either case would be unusual for a chloroplastic protein (Keegstra et al. 1989; Berry-Lowe and Schmidt 1991).

As a first step toward engineering glycine betaine biosynthesis, we introduced cDNA sequences encoding spinach or beet BADH into tobacco (*Nicotiana tabacum* L.). We believed – and during this work confirmed – that it is prudent to introduce BADH before choline monooxygenase because betaine aldehyde is toxic to plants. Tobacco was chosen as the target species because it has been shown to lack activity for both steps in the glycine betaine biosynthesis pathway (Weretilnyk et al. 1989). We wished to know (i) if the transgene product would be targeted to the appropriate (chloroplastic) compartment and would be enzymically active; (ii) if transgenic plants would accumulate glycine betaine when supplied with the intermediate betaine aldehyde; and (iii) if BADH would confer resistance to betaine aldehyde toxicity.

Materials and methods

Plasmids and bacteria. Standard techniques were used for plasmid constructions (Sambrook et al. 1989). To create BADH 1 to 5, BADH cDNAs from spinach (in pBlueScript) (Weretilnyk and Hanson 1990) or from beet (in pUC119) (McCue and Hanson 1992) were subcloned into the expression cassette of pGA643 (An et al. 1988). The pGA643 cassette has the cauliflower mosaic virus (CaMV) 35S promoter and the transcript 7 and 5 terminators of Ti plasmid pTiA6. Individual constructs in pGA643 were: BADH 1, SmaI/ClaI fragment containing 2028 base pairs (bp) of spinach BADH cDNA, vector sequences of 9 bp at the 5′ and approximately 207 bp at the 3′ end, cloned into the HpaI site; BADH 2, BamHI/BclI fragment containing 1584 bp of spinach BADH cDNA and 17 bp vector sequence at the 5′ end, in the BglII site; BADH 3, EcoRI fragment of beet BADH cDNA D in the HpaI site; BADH 4, Eco RI/BamHI fragment of beet cDNA D in the HpaI site; BADH 5, BamHI fragment of beet BADH cDNA A in the BglII site. The cDNAs in BADH 1, 3 and 4 included the poly(A) tail; BADH 2 and 5 did not. Plasmids were transferred to *Agrobacterium tumefaciens* LBA4404 by direct transformation (An et al. 1988).

Plant transformation and genetic analyses. Leaf disks were taken from *Nicotiana tabacum* L. cv. Havana 38 (= Wisconsin 38) plants cultured on MS medium (Murashige and Skoog 1962) with 3% (w/v) sucrose, 0.1 $\mu g \cdot ml^{-1}$ kinetin, 0.05 $\mu g \cdot ml^{-1}$ indoleacetic acid and 0.7% (w/v) phytagar (GIBCO-BRL, Gaithersburg, Md., USA). Leaf-disk transformation, and selection and propagation of kanamycin-resistant plants were as described by An et al. (1988). Standard techniques were used for self-pollination and for scoring seedlings for kanamycin resistance (Budar et al. 1986). Segregation of BADH was tested on the youngest expanded leaf of four-week-old plants.

Enzyme extraction and analyses. Extraction of leaf proteins and $(NH_4)_2SO_4$ precipitation were as previously reported (Weretilnyk et al. 1989). Protein was determined by the Bradford method (Bradford 1976). NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed in the reductive direction (Wolosiuk and Buchanan 1976); the enzyme was activated by preincubating in 0.1 M N-tris[hydroxymethyl]methylglycine-NaOH (pH 8.4), containing 10 mM dithiothreitol (DTT), 5 mM ATP and 20 mM Na-phosphate for 5 min at 25°C. Assays for BADH (Weretilnyk and Hanson 1989), catalase (Luck 1965) and fumarase (Racker 1950) were as described except that the assay volume was 100 µl. Methods for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, native polyacrylamide gel electrophoresis (PAGE), and staining of gels for BADH activity were as given previously (Weigel et al. 1986; Weretilnyk and Hanson 1989).

Northern blot analysis. Total RNA was isolated from leaves by the method of Puissant and Houdebine (1990). Samples of total RNA (20 µg) were separated by formaldehyde (8.1%)/agarose (1.5%) gel electrophoresis and blotted to nitrocellulose (Sambrook et al. 1989). Blots were hybridized with a 1584-bp spinach cDNA (Weretilnyk and Hanson 1990) labeled with ^{32}P to a specific activity of approximately 10^8 cpm·(µg DNA)$^{-1}$ using a random-primed DNA labeling kit (Boehringer Mannheim Canada, Laval, Québec). Conditions used for hybridization, washing and autoradiography were as described previously (McCue and Hanson 1992).

Metabolism of deuterium-labeled betaine aldehyde by leaf disks. Batches of eight disks (11 mm diameter) from fully expanded leaves of eight-week-old plants were infiltrated in 5 ml of 1 mM betaine aldehyde (methyl-d_3) and incubated (pH 6.5) for 6 h in the light as described by Weretilnyk et al. (1989). Methods used for preparation of d_3-betaine aldehyde (Rhodes et al. 1987), for ion-exchange separation of glycine betaine and betaine aldehyde, and for determination of these compounds by fast atom bombardment-mass spectrometry (FAB-MS) of their *n*-butyl derivatives were as reported previously (Rhodes et al. 1987).

Chloroplast isolation and purification. Mesophyll protoplasts were isolated from destarched leaves of 1- to 12-week-old plants by incubating 2-mm strips (1 g FW per 10 ml) in a 1:1 (v/v) mixture of enzyme solution and KM8p medium (Kao and Michayluk 1975) for 16 h at 25°C under fluorescent lamps (photon flux density 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The enzyme solution contained 2% (w/v) Cellulase R-10, 0.2% (w/v) Macerozyme R-10, (both from Yakult Honsha Co., Tokyo, Japan), 6.8 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 0.7 M glucose and 3 mM 2-[N-morpholino]ethanesulfonic acid (Mes)-KOH, pH 6.5. Protoplasts were purified by flotation on 20% (w/v) sucrose (Rathinasabapathi and King 1992). Operations were at 0–4°C. Organelles were released by resuspending protoplasts (approx. 1 mg chlorophyll·ml^{-1}) in isotonic medium (0.54 M sorbitol; 1 $mg \cdot ml^{-1}$ bovine serum albumin (BSA); 5 mM Na-ascorbate; 10 mM DTT; 2 mM Na_2EDTA in 50 mM Hepes-KOH, pH 7.6) and passing through a 15- µm nylon mesh. Broken protoplasts were layered on 10 ml of 40% (w/v) Percoll in 50 mM Hepes-KOH (pH 7.6) containing 0.54 M sorbitol, 5 mM DTT, 1 mM Na_2EDTA and 1 $mg \cdot ml^{-1}$ BSA. Chloroplasts were pelleted by centrifuging at 3920·*g* for 2 min, and rinsed with isotonic medium. Chloroplasts or protoplasts were lysed by shaking in lysis medium (5 mM Na-ascorbate; 10 mM DTT; 5 mM $MgCl_2$; 2 mM Na_2EDTA in 50 mM Hepes-KOH, pH 7.6). Protoplasts were then sonicated for 3 × 10 s. Stromal and protoplast lysate fractions were then obtained by centrifuging lysed chloroplasts or protoplasts for 10 min at 16000·*g*, followed by desalting on Sephadex G-25 equilibrated with 50 mM Hepes-KOH (pH 8.0) and 1 mM Na_2EDTA. Chloroplast intactness (Lilley et al. 1975) and chlorophyll (Arnon 1949) were determined by published methods.

Tests for toxicity of betaine aldehyde. Leaf disks (15 mm diameter) were cultured in continuous light (photon flux density 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on MS medium supplemented with 1 $\mu g \cdot ml^{-1}$ 6-benzyladenine,

3% (w/v) sucrose, 0.7% (w/v) phytagar and 0–10 mM betaine aldehyde. Seeds were placed on medium containing MS salts, 10 mM Mes-KOH (pH 5.8) and 0.7% (w/v) phytagar and 0–10 mM betaine aldehyde, and incubated in continuous light.

Metabolism of [^{14}C]*glycine betaine.* [Methyl-^{14}C]glycine betaine (2.1 GBq·mmol^{-1}) was synthesized from [methyl-^{14}C]choline (DuPont-NEN Canada, Mississauga, Ontario) using choline oxidase (Sigma, St. Louis, Mo., USA). [Methyl-^{14}C]methionine (1.8 GBq·mmol^{-1}) was from DuPont-NEN. Tobacco cv. Xanthi plants were cultured in MS medium containing 75 kBq of [^{14}C]glycine betaine or [^{14}C]methionine for four weeks in continuous light. Extraction of shoots, separation of metabolites by ion exchange, TLC, and autoradiography were as described previously (Ladyman et al. 1980). For analysis of nicotine, alkaline extracts of dried leaves (Cromwell 1955) were used.

Results

Levels of BADH enzyme, protein and mRNA. Tobacco plants were transformed using five BADH constructions cloned into the plant expression vector pGA643, which contains the CaMV 35S promoter. Constructions differed in the source of the BADH cDNA sequence (spinach or beet) and in whether or not the poly(A) tail and part of the 3′ untranslated region had been deleted. Putative transformants were selected based on kanamycin resistance, and screened for BADH activity in leaf extracts. Wild-type tobacco or plants transformed with the expression vector alone showed slight endogenous BADH activity (< 1 nmol·min^{-1}·(mg protein)$^{-1}$), as reported previously for wild-type tobacco (Weretilnyk et al. 1989). Of 78 independent transformants screened, 48 had elevated BADH activities; these included several representatives of each BADH construction. Their BADH enzyme levels were in all cases comparable to or above those found in salinized spinach leaves (Fig. 2). There was significant variation in BADH expression among constructions, but it was not related in a simple way to the removal of 3′ untranslated sequences because this gave opposite effects for spinach and beet genes. Segregation for kanamycin resistance and BADH indicated the Mendelian inheritance of these genes, with one, two or ≥ three loci (data not shown).

Immunoblot analysis confirmed that all transgenic plants with BADH activity contained a BADH monomer with the same molecular weight as that from spinach (Fig. 2, inset). Wild-type plants and those transformed with the vector alone had very little immunoreactive peptide. Because electrophoresis in non-denaturing gels can reveal subtle differences in mass/charge ratio (Hedrick and Smith 1968), leaf proteins were separated by native polyacrylamide gel electrophoresis and stained for BADH activity. Activity bands with the same mobility as spinach or beet BADH were found in transgenic plants, according to the gene that they expressed (Fig. 3). There were no bands in vector-alone controls. The K_m for betaine aldehyde for the BADHs of three plants transformed with spinach genes was in the range 50–70 μM, which was the same as that found for BADH from spinach leaves (data not shown).

Northern blot analysis showed that tobacco plants transformed with spinach or beet genes expressed a

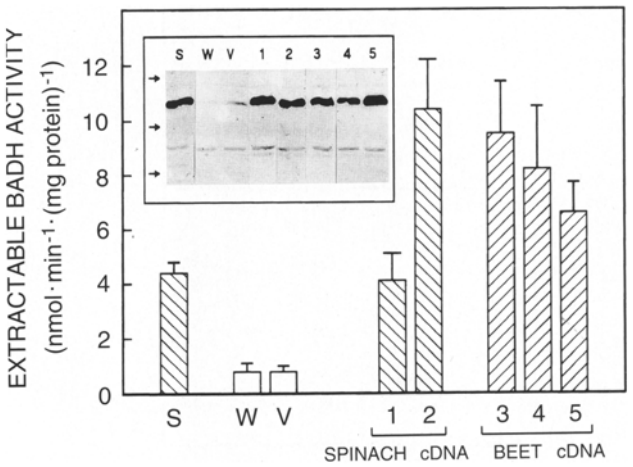

Fig. 2. Activities of BADH enzyme and levels of BADH protein in leaves of transgenic tobacco. Proteins were extracted from expanding leaves of plants eight to ten weeks old and used for enzyme assays (*bar graphs*) or SDS-PAGE and immunoblot analysis (*inset*). Bar-graph data are means and SE for 4 individual untransformed control tobacco plants (*W*), for 8 independent transformants with the vector pGA643 alone (*V*), and for 6–17 independent transformants for each of the BADH cDNA constructions (1–5). Data for eight extracts of spinach plants salinized for one week with 200 mM NaCl (*S*) are included for comparison. Immunoblot data are for single representative plants of each type; 50 μg of leaf proteins was loaded per lane. *Arrows* left of the immunoblots indicate positions of molecular-weight standards (80, 49.5 and 32.5 kDa, in order from the top)

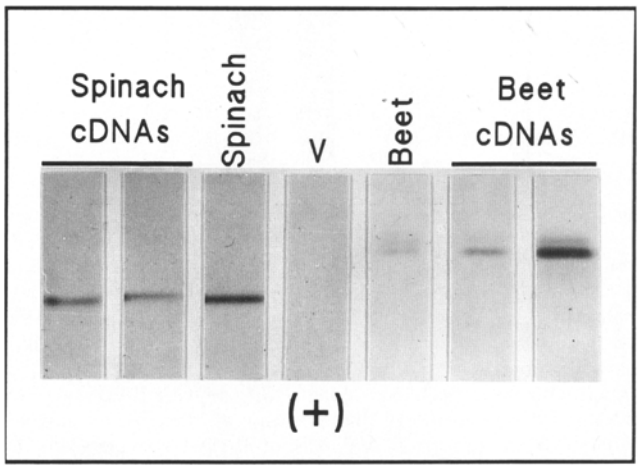

Fig. 3. Analysis of BADH enzymes from transgenic tobacco by native PAGE. Leaf proteins precipitated with ammonium sulfate (1.8–2.6 M) were separated by native PAGE and stained for BADH activity. Lanes contained 0.4–0.5 mg protein from four representative tobacco plants expressing spinach or beet cDNAs (BADH 2 or 3), from spinach and beet plants, and from tobacco plants transformed with the vector pGA643 alone (*V*)

BADH mRNA of the same size (1900 bp) as those in spinach and beet plants (Fig. 4a). Consistent with the cDNAs being under the control of a constitutive promoter, applying salt stress had little effect on either BADH mRNA level (Fig. 4a) or enzyme activity (Fig. 4b).

Subcellular localization of BADH. To determine whether the BADH enzyme is correctly targeted to chloroplasts

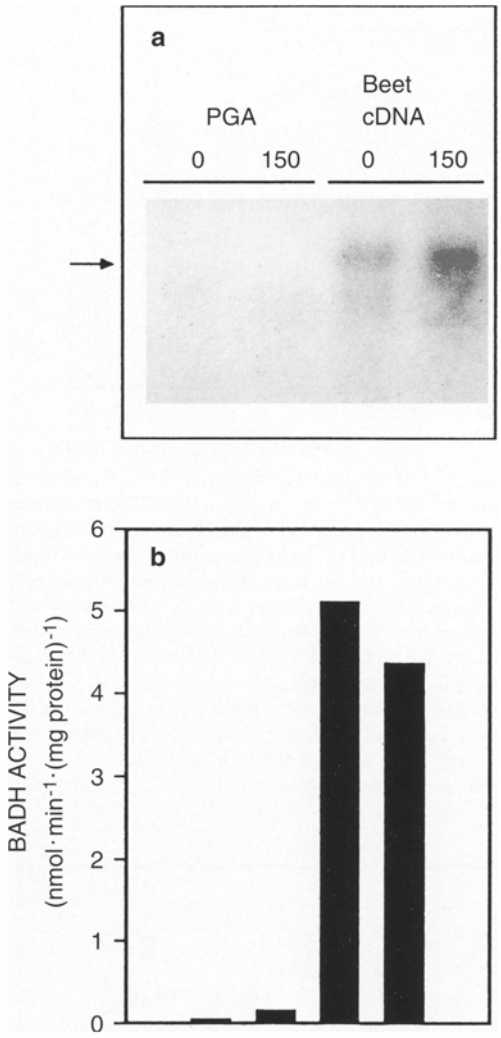

Fig. 4.a,b. Expression of BADH mRNA and BADH enzyme activity in control and salt-stressed transgenic tobacco plants. Plants were irrigated with mineral nutrient solution containing no NaCl (0) or a final concentration of 150 mM NaCl (150). Salt-stress was applied by raising the NaCl concentration in 50-mM steps each 3 d, and maintaining the final concentration for 6 d. **a** Northern blot analysis of total RNA (20 μg) from leaves of tobacco plants transformed with vector pGA643 alone (*PGA*) or with BADH 4 (*Beet cDNA*). The *arrow* marks the position of the BADH mRNA (1900 bp) from spinach. **b** Activities of BADH enzyme from the leaves of the same plants as in **a**

in transgenic tobacco, intact chloroplasts were prepared from protoplast lysates, and purified by centrifugation through Percoll. Table 1 gives results from two such experiments. The purifed chloroplasts were little contaminated by mitochondria or peroxisomes, as judged by fumarase and catalase markers. The recovery of BADH in the stromal fraction was comparable to that of the stromal marker enzyme, GAPDH. Centrifugation of this fraction at 135 000·*g* for 90 min did not pellet BADH activity, establishing that the BADH enzyme was not associated with membranes. The chloroplastic localization of BADH was confirmed in eight further experiments like those of Table 1, using plants chosen at various ages (Fig. 5). As expected, the relative levels of BADH and GAPDH activity varied considerably, reflecting differences in the developmental stage of the seedling leaves used to make protoplasts and differences between individual transformants. The BADH/GAPDH ratios in the purified chloroplasts were highly correlated with those in the corresponding protoplast preparations ($r^2 = 0.99$, $P < 0.001$); the mean value for BADH recovery in the stromal fraction calculated from all ten experiments was 97.2% (SE ± 8.3%). It should be noted that the native BADH dimer proved to be resistant to several proteases (trypsin, chymotrypsin, thermolysin and proteinase K). This made it impractical to use protease-protection criteria to establish a chloroplast location in this instance. More specifically, since trypsin and chymotrypsin did not digest BADH, our data do not exclude the possibility that BADH is localized in the chloroplast intermembrane space.

In-vivo oxidation of d_3-betaine aldehyde to d_3-glycine betaine. Expressing BADH in tobacco chloroplasts might not be sufficient to confer the capacity to oxidize supplied betaine aldehyde if tobacco lacks the systems required to transport betaine aldehyde across the plasmalemma or chloroplast envelope. To investigate this, leaf disks of transgenic plants and controls were given substrate amounts of d_3-betaine aldehyde, and its uptake and oxidation to d_3-glycine betaine were measured by FAB-MS (Fig. 6). Vector-alone controls took up d_3-betaine aldehyde quite readily but oxidized little of it. Transgenic tissues showed higher rates of uptake and substantial oxidation; oxidation rates were comparable to those re-

Table 1. Evidence for chloroplastic localization of BADH in transgenic tobacco. Protoplasts were isolated from progeny of primary transformants expressing spinach or beet cDNAs (BADH 2 or 4). Chloroplasts were >95% intact before lysis. Activities of BADH and marker enzymes were assayed in protoplast lysates and corresponding stromal fractions of Percoll-purified chloroplasts. Enzyme activities are given relative to chlorophyll

cDNA	Enzyme	Units	Enzyme activity (units·min^{-1}·(mg chlorophyll)$^{-1}$)	
			Protoplast lysate	Stromal fraction
Spinach	GAPDH	nmol	2520	2130
	BADH	pmol	2490	2560
	Fumarase	nmol	46	<6
	Catalase	μmol	111	<5
Beet	GAPDH	nmol	2710	2630
	BADH	pmol	10300	9400
	Fumarase	nmol	104	<18
	Catalase	μmol	26	<3

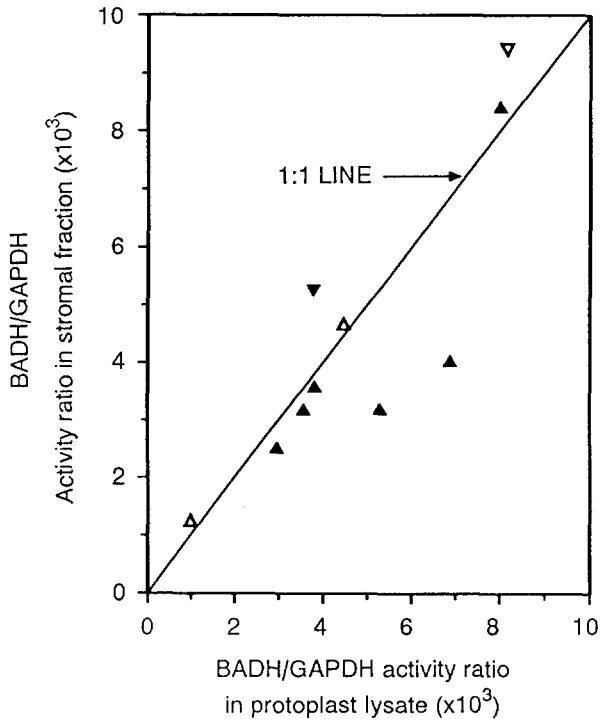

Fig. 5. Evidence that BADH is located in the chloroplasts of transgenic tobacco plants of various ages. Protoplasts were prepared from the leaves of progeny of primary transformants, 1–12 weeks of age. The relative activities of BADH and the stromal marker GAPDH were determined for each protoplast preparation and for the corresponding chloroplast stromal fraction. Plants were expressing spinach BADH 2 (△, ▽) or beet BADH 3 or 4 (▲, ▼). For BADH/GAPDH ratios >10, the ratios were multiplied × 0.1 (*inverted symbols*)

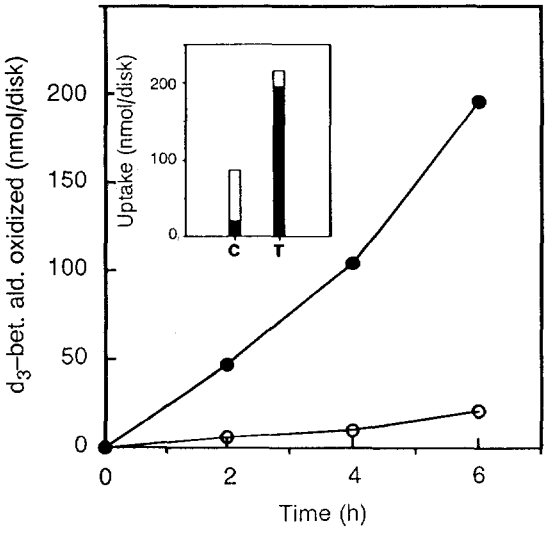

Fig. 6. Uptake and oxidation of d_3-betaine aldehyde by leaf disks of transgenic tobacco plants. Plants were primary transformants carrying the pGA643 vector alone (○) or beet BADH 3 (●). Leaf disks were taken from fully expanded leaves of eight-week-old plants. The *bar graphs* in the *inset* show the uptake of d_3-betaine aldehyde at 6 h by control (*C*) or transgenic (*T*) disks. The *shaded areas* show the proportion of d_3-betaine aldehyde taken up which had been oxidized to d_3-glycine betaine

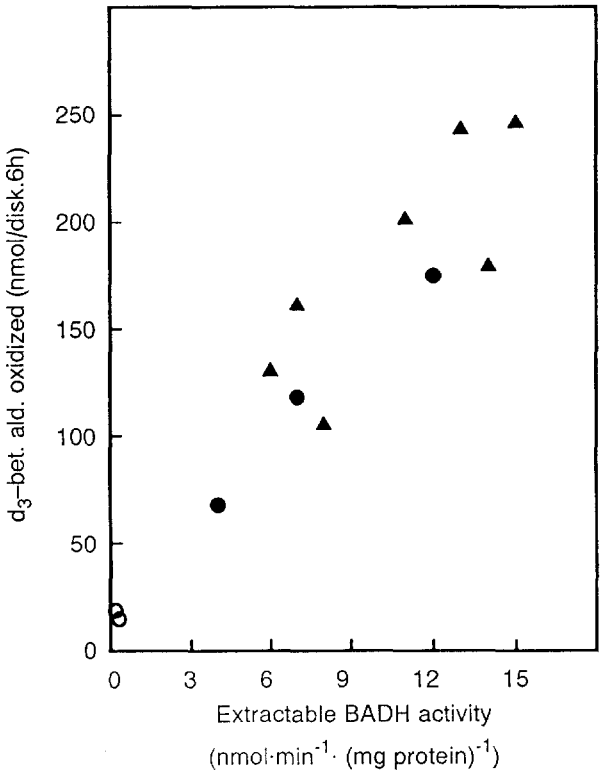

Fig. 7. Relationship between the level of extractable BADH activity and the in-vivo rate of oxidation of d_3-betaine aldehyde by tobacco leaf disks. Fully expanded leaves from the same eight-week-old plants were used for enzyme assays and for cutting disks. ○, wild-type plants or plants transformed with the vector pGA643 alone; ●, plants transformed with spinach cDNAs (BADH 1 and 2); ▲, plants transformed with beet cDNAs (BADH 3, 4 and 5)

ported for spinach (Rhodes et al. 1987; Weretilnyk et al. 1989). For various independent transformants, the in-vivo rates of oxidation of d_3-betaine aldehyde to glycine betaine were significantly correlated ($r^2 = 0.77$, $P < 0.05$) with extractable BADH activity (Fig. 7). Such a correlation would not be expected if transport of betaine aldehyde was rate-limiting.

Betaine aldehyde-resistant phenotype. Because small aldehydes such as betaine aldehyde are generally toxic whereas glycine betaine is benign, tobacco tissues expressing BADH might be expected to be resistant to betaine aldehyde. This idea was tested using regenerating leaf disks and germinating seedlings. When wild-type leaf disks were cultured on shoot-regeneration medium containing betaine aldehyde they were severely inhibited, whereas disks expressing the beet transgene regenerated quite normally. This effect was most marked at 5 mM betaine aldehyde (Fig. 8) but was also apparent at 2 and 10 mM. Similarly, 5 or 10 mM betaine aldehyde depressed cotyledon expansion and greening much more strongly in control seedlings than in those expressing beet BADH (Fig. 9a). To verify that these effects were due to in-vivo conversion of betaine aldehyde to glycine betaine, seedlings cultured with betaine aldehyde were ana-

Fig. 8. Effect of betaine aldehyde on shoot regeneration from tobacco leaf disks. Disks from wild-type tobacco plants (*upper row*) or plants transformed with beet cDNA construction BADH 3 (*lower row*) after culture for four weeks on medium containing 5 mM betaine aldehyde

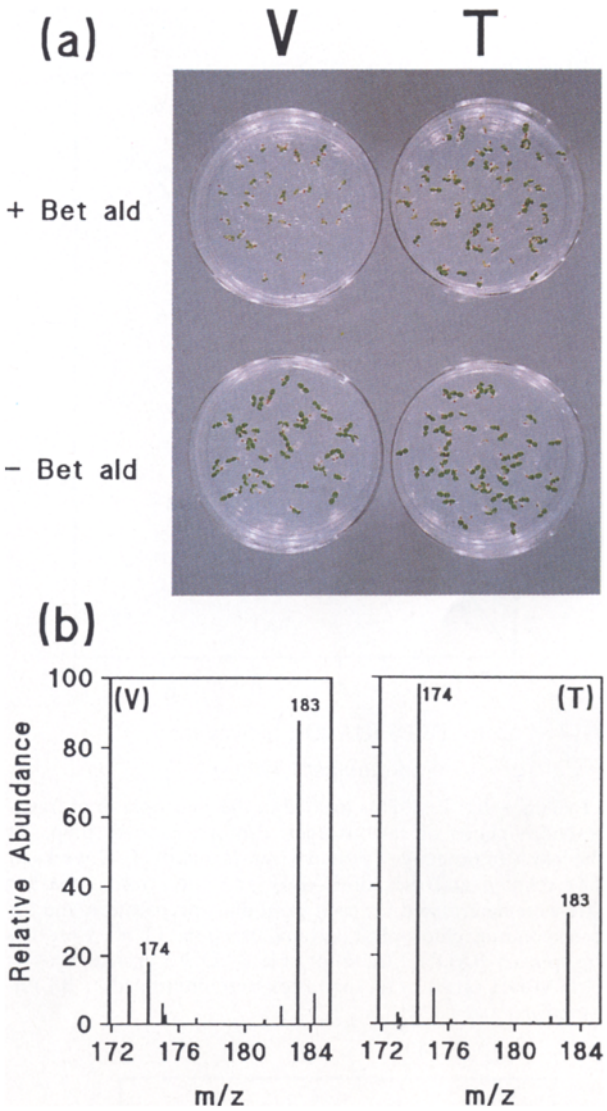

Fig. 9a,b. Betaine-aldehyde resistance and glycine-betaine accumulation. **a** Progeny of tobacco plants transformed with the vector pGA643 alone (*V*) or with beet cDNA BADH 4 (*T*), germinated for 7 d on mineral nutrient medium with or without 5 mM betaine aldehyde. The variability of progeny population T on betaine-aldehyde medium can be attributed to segregation. **b** The FAB mass spectra for the cationic fractions of tobacco seedlings transformed with pGA643 alone (*V*) or with beet cDNA BADH 4 (*T*), cultured for 14 d on mineral nutrient medium containing 5 mM betaine aldehyde. Cation fractions were isolated from shoots of 35 seedlings, spiked with an internal standard of 448 nmol of d_9-glycine betaine, and derivatized with *n*-butanol. Note the relative sizes of the peaks at m/z 183 (molecular ion of d_9-glycine betaine *n*-butyl ester) and m/z 174 (molecular ion of endogenous glycine betaine *n*-butyl ester)

lyzed by FAB-MS. Those expressing beet BADH had betaine aldehyde levels of only 4.0 μmol·(g FW) ^{1} while controls carrying the vector alone had 16.4 μmol·(g FW)$^{-1}$. Their respective levels of glycine betaine were 20.4 and 2.4 μmol·(g FW)$^{-1}$ (Fig. 9b). Only a trace of glycine betaine (<0.1 μmol·(g FW) ^{1}) was present in seedlings cultured without betaine aldehyde (not shown).

Accumulation of glycine betaine. The large potential for accumulation of glycine betaine in transgenic seedlings exposed to betaine aldehyde is illustrated by the mass spectra of Fig. 9b. Such large accumulations imply that tobacco has little ability to metabolize glycine betaine, as in barley (Ladyman et al. 1980) and beet (Hanson and Wyse 1982). We confirmed this experimentally by culturing sterile tobacco plants on medium containing [methyl-^{14}C]glycine betaine of high specific radioactivity. After four weeks, >95% of the ^{14}C label was absorbed; no labeled compounds besides glycine betaine were detected within the plants. We specifically checked for ^{14}C-incorporation into nicotine because Byerrum et al. (1956) reported that glycine betaine is a methyl donor for nicotine biosynthesis in tobacco. Although nicotine did not acquire label from [^{14}C]glycine betaine, it did so readily (to a specific activity of 0.19 MBq·mmol^{-1}) in positive control experiments in which [methyl-^{14}C]methionine replaced [^{14}C]glycine betaine. The results of Byerrum et al. (1956) probably reflect the activities of microorganisms present under the non-sterile culture conditions used (Wyn Jones et al. 1973).

Discussion

Transgenic tobacco lines expressing an active chloroplastic BADH represent the first step in metabolic engineering of the glycine betaine biosynthesis pathway. The main interest of this metabolic engineering relates to drought and salt stress. We therefore verified that the constructions used would give satisfactory BADH expression under stress conditions. The next step in the metabolic engineering process will be to introduce genes coding for choline monooxygenase, which is a chloroplastic enzyme in spinach (Brouquisse et al. 1989). Be-

cause the product of choline monooxygenase – betaine aldehyde – is toxic, we sought to obtain transgenic plants expressing BADH in chloroplasts so that this enzyme would have immediate access to its substrate. It was not evident at the outset that the protein products of spinach and beet BADH cDNA sequences would be targeted to chloroplasts in tobacco. Neither sequence encodes a recognizable transit peptide; spinach BADH may have no transit peptide at all, or at most an exceptionally short one of eight residues or fewer. Consistent with this, the in-vitro translation products of BADH mRNAs differ little if at all in molecular weight from the mature BADH subunits (McCue and Hanson 1992; Weretilnyk and Hanson 1989).

Despite the persuasive evidence against a cleavable transit peptide of typical size and composition, our data show that spinach and beet BADHs were efficiently (>90%) directed to the chloroplasts in transgenic tobacco. The spinach or beet BADH enzymes expressed in tobacco chloroplasts were indistinguishable from the BADH enzymes isolated from spinach or beet, with respect to molecular weight and mass/charge ratio. Thus, if cleavage of a short transit peptide or other processing events occur in spinach and beet, it is likely that they occur in tobacco also. These results suggest two possibilities. There could be a very short, cleavable transit sequence in spinach and beet BADHs. Alternatively, as for a 22-kDa heat-shock protein of *Chlamydomonas* (Grimm et al. 1989), the targeting information for BADH could be in the mature polypeptide itself. These alternatives might be distinguished by in-vitro experiments on the uptake of BADH precursors using spinach chloroplasts. By labeling the BADH precursor with [^{35}S]methionine, and carrying out peptide mapping of the product imported into chloroplasts, cleavage or modification of the N-terminal eight-amino-acid tryptic fragment would be apparent.

Quaternary ammonium compounds such as betaine aldehyde most probably depend on specific transport systems to cross plant cell membranes, as in bacterial cells (Csonka and Hanson 1991). A priori there seemed no reason for such systems to exist in tobacco, except perhaps that certain other Solanaceae accumulate glycine betaine (Weretilnyk et al. 1989). However, transgenic tobacco absorbed and oxidized exogenous betaine aldehyde at high rates, showing that betaine aldehyde transport across the plasmalemma and chloroplast envelope is as effective as in spinach.

When supplied with betaine aldehyde, transgenic tobacco plants accumulated glycine betaine to levels similar to those found in salinized or drought-stressed plants (Rhodes and Hanson 1993). It would be interesting to test whether this glycine betaine affords tolerance to osmotic stress. Unfortunately, the toxicity of betaine aldehyde complicates the interpretation of such experiments. Although transgenic plants metabolize betaine aldehyde at rates sufficient to confer resistance to this compound, their growth is still retarded compared to controls. The capacity to detoxify betaine aldehyde may be of practical interest in itself. Drug- and herbicide-resistance genes have environmental drawbacks as selectable markers for plant transformation (Flavell et al. 1992). Resistance to betaine aldehyde could be a viable alternative for crops which lack glycine betaine. These include tomato, potato, rice, cucurbits, brassicas and most legumes.

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