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Expression of bacterial L-aspartate-α-decarboxylase in tobacco increases β-alanine and pantothenate levels and improves thermotolerance★

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Abstract

L-Aspartate-α-decarboxylase catalyzes the decarboxylation of L-aspartate to generate β-alanine and carbon dioxide. This is an unusual pyruvoyl-dependent enzyme unique to prokaryotes that undergoes limited self-processing. The *Escherichia coli panD* gene encoding L-aspartate-α-decarboxylase was expressed under a constitutive promoter in transgenic tobacco. Transgene expression was verified by assays based on RNA blots, immunoblots and enzyme activity *in vitro*. The *panD* lines had increased levels of leaf β-alanine (1.2- to 4-fold), pantothenate (3.2- to 4.1-fold) and total free amino acids (up to 3.7-fold) compared to wild-type and vector controls. Growth of homozygous lines expressing *E. coli* L-aspartate-α-decarboxylase was less affected than that of the control lines when the plants were stressed for 1 week at 35 °C. When transferred from 35 to 30 °C for 3 weeks, the *PanD* transgenic lines recovered significantly ($P \leq 0.001$) better than the controls: *PanD* lines had on an average 54% and 84% greater fresh and dry weights respectively, compared to the controls. Homozygous lines expressing *E. coli* L-aspartate-α-decarboxylase had significantly greater thermotolerance ($P \leq 0.05$) during germination. At 42 °C, 95% of two T3 *PanD* transgenic line seeds germinated after 12 days compared to 73% for the wild-type seeds. Our results indicated that *E. coli* L-aspartate-α-decarboxylase was correctly processed and active in the transgenic eukaryotic host and its expression resulted in increased thermotolerance in tobacco.

Introduction

β-Alanine (β-ala), the non-protein amino acid, is found in all living organisms and essential for normal growth because it is a precursor for vitamin B5, pantothenate (Raman and Rathinasabapathi, 2004). Bacteria and yeast mutants deficient in β-ala biosynthesis are lethal (Cronan, 1980; Merkel and Nichols, 1996; White *et al.*, 2001). Other than its vital role, β-ala has also been implicated in plant adaptation to environmental stress. In a cowpea cell culture, β-ala level increased 2-fold within 4 h and more than 5-fold after 24 h of heat shock (Mayer *et al.*, 1990). In *Arabidopsis*, β-ala level increased significantly when plants were exposed to either heat stress and/or drought stress (Kaplan *et al.*, 2004; Rizhsky *et al.*, 2004). In a recent study, β-ala was found to suppress the heat-induced inactivation of lactate dehydrogenase (LDH) *in vitro* (Mehta and Seidler, 2005). Fitting to a chemical chaperone, addition of β-ala also prevented aggregation of LDH and reactivated thermally denatured LDH (Mehta and Seidler, 2005).

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In most members of the plant family Plumbaginaceae, β-ala is methylated to the osmoprotectant β-ala betaine (Rathinasabapathi *et al.*, 2001; Raman and Rathinasabapathi, 2003). In some legumes, β-ala along with γ-glutamate and cysteine forms homoglutathione, a thiol tripeptide involved in heavy metal detoxification and protection against reactive oxygen species (Klapheck *et al.*, 1988; Moran *et al.*, 2000).

Despite its central role in pantothenate synthesis and in environmental stress adaptation, β-ala biosynthesis in plants has not been characterized well. However, in *Escherichia coli* it is synthesized *via* α-decarboxylation of L-aspartate catalyzed by the *panD*-encoded L-aspartate-α-decarboxylase (ADC, Williamson and Brown, 1979; Merkel and Nichols, 1996). This route appears to be unique to prokaryotes and absent in eukaryotes including yeast and plants (Rathinasabapathi *et al.*, 2000; White *et al.*, 2001). *E. coli* ADC is an unusual enzyme because of its requirement for pyruvate as a covalently bound, catalytically active prosthetic group (Williamson and Brown, 1979, Ramjee *et al.*, 1997). The enzyme is initially translated as an inactive precursor protein (π-protein, 13.8 kD). It undergoes self-processing at a specific Gly_{24}–Ser_{25} bond to generate a β-subunit (2.8 kD) with hydroxyl group at its C-terminus and an α-subunit (11.0 kD) with a pyruvoyl group at its N-terminus, derived from serine (Ramjee *et al.*, 1997; Albert *et al.*, 1998). The crystal structure of the *E. coli* ADC demonstrated that the active enzyme is a multimer containing three each of α- and β-subunits and an incompletely processed π-protein (Albert *et al.*, 1998).

Metabolic engineering for β-ala overproduction in plants could potentially be useful in engineering plants tolerant to environmental stress in addition to increasing pantothenate, for improved nutritional value and for engineering plants for heavy metal detoxification. Also, creation of transgenic plants with elevated levels of β-ala is an important first step prior to generating transgenic plants capable of accumulating osmotically significant quantities of β-ala betaine *via* the expression of *Limonium latifolium* β-ala *N*-methyltransferase (Raman and Rathinasabapathi, 2003). However, plant genes involved in β-ala synthesis have not been characterized yet (Raman and Rathinasabapathi, 2004). We therefore aimed to use *E. coli* *panD* gene encoding ADC for engineering tobacco since the bacterial route to β-ala is a well-characterized single step decarboxylation of aspartate and not subject to feed back inhibition by metabolic end products β-ala, pantothenate, acetyl coenzyme A and coenzyme A (Williamson and Brown, 1979; Cronan, 1980). Since *E. coli* ADC protein's processing and activity *in vitro* are stimulated by high temperature (Ramjee *et al.*, 1997), we examined the effect of transgene expression on the transgenic plant's thermotolerance. Expression of *E. coli* *panD* gene in transgenic tobacco resulted in increased thermotolerance.

Experimental procedures

Materials

Bacterial media, antibiotics, buffers, protease inhibitors and DEAE-Sepharose were from Sigma (St. Louis, MO). Plasmid purification and gel extraction kits were from Qiagen (Valencia, CA). L-[U-^{14}C]aspartate (217 mCi mmol^{-1}) from ICN Biomedicals (Irvine, CA) and [α-^{32}P]GTP, 800 Ci mmol^{-1}) from Amersham Bioscience (Piscataway, NJ) were used without further purification. Protein molecular weight markers, protein stains and PVDF membranes were from BioRad (Hercules, CA). Bacterial expression vector pET-Blue-2 and *E. coli* BL21-DE3 were from Novagen (Madison, WI). DNA M_r marker, *Taq* polymerase, dNTPs, restriction enzymes, pCR 2.1-TOPO cloning kit, TRIzol reagent, Pro-Bond Ni-NTA resin, and secondary antibodies were from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by the custom primer synthesis unit of Invitrogen (Carlsbad, CA).

Construction of the expression vector

E. coli panD open reading frame (ORF) with the start and stop codon (429 bp) was amplified from $DH_5\alpha$ genomic DNA using the primers 5′–3′ CCGAGCTCGACAGGGTAGAAAGGTAGA and CCCCATGGGGGATAACAATCAAGCAA CC. The PCR product, cloned in pCR 2.1-TOPO vector, was verified by sequencing. A single point mutation in the clone's ORF converted Cys_{26} to Tyr. This *panD* gene was sub-cloned in the right frame into pUC-18 vector under *lac* promoter. The pUC-*panD* vector successfully complemented an

E. coli mutant defective in β-ala synthesis (ATCC Number AB354), confirming that the cloned gene coded for an active ADC.

The plant expression vector pMON979 (Monsanto Chemical Company, St. Louis, MO) contains a multiple cloning site (MCS) between an enhanced CaMV 35S promoter and NOS3′ terminator, a kanamycin resistance selectable marker for plant selection and a spectinomycin resistance gene for bacterial selection. A 34-bp synthetic dsDNA containing *Bam*HI, *Sac*I, *Hpa*I, *Apa*I, *Kpn*I and *Eco*RI sites was ligated into the *Bgl*II and *Eco*RI sites of the MCS to create pMON-R5. *E. coli panD* ORF in TOPO-*panD* vector was digested with *Eco*RI and sub-cloned into *Eco*RI digested pMON-R5 plant expression vector to derive pMON-R5-*panD*. Recombinants with the insert in the correct orientation were identified by restriction analyses.

Agrobacterium-mediated transformation of tobacco

The pMON-R5-*panD* and pMON-R5 were transferred into *Agrobacterium tumefaciens* ABI strain *via* triparental mating (An *et al*., 1988). Transformation of *Nicotiana tabacum* cv. Havana 38 (Wisconsin 38) was performed as described previously (An *et al*., 1988; Rathinasabapathi *et al*., 1994). Putative transformants were selected based on their resistance to kanamycin (50 mg l^{-1}) in the media and verified by PCR using *panD* specific primers.

DNA and RNA blot analyses

Genomic DNA (20 μg), extracted using CTAB method (Wong and Taylor, 1993), was digested with restriction enzymes, separated by 1.2% (w/v) agarose gel, and transferred to nylon membranes (Sambrook *et al*., 1989). Total leaf RNA (20 μg), extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), was separated in formaldehyde 1.2% (w/v) agarose gel, and transferred to nylon membrane (Sambrook *et al*., 1989). Equal loading of RNA was verified by ethidium bromide staining. A 446 bp of *PanD* sequence was labeled with [^{32}P]dGTP (800 Ci mmol^{-1}, Amersham Bio-Sciences) using a random primer method (Invitrogen) according to manufacturer's instructions. Hybridization, membrane washing and autoradiography were done as described (Raman and Rathinasabapathi, 2003).

Genetic analyses and identification of homozygous lines

Primary transgenic lines and wild-type (WT) tobacco were grown in a greenhouse and selfed. Surface sterilized T_2 seeds of each line were placed on Petri plates containing half-strength MS salts media supplemented with kanamycin (200 mg l^{-1}). Seeds were germinated at 24 °C under continuous light (40–50 μmol m^{-2} s^{-1}) for 14 d. Seedlings were scored for their resistance to kanamycin and segregation was analyzed using a X^2 test. Several T_2 lines with single gene segregation for kanamycin resistance were grown in a greenhouse for flowering and their seeds were analyzed for the segregation of the marker. Those lines that did not segregate at T3 generation were considered homozygous.

ADC expression, purification and polyclonal antibodies

The *panD* gene was amplified from *E. coli* DH$_5$α genomic DNA using primers 5′–3′ TCATGATT CGCACGATGCTGCCAGG and CAGCTGAG CAACCTGTACCGGAATCGC primers. The *Bsp*HI site was introduced on the *panD* ATG start codon by the forward primer and the *Pvu*II site was introduced at the 3′-end by the reverse primer. The PCR product, generated using Advantage-HF 2 PCR Kit (Clontech, Palo Alto, CA) was digested with *Bsp*HI and *Pvu*II and ligated directly into *Nco*I, *Pvu*II digested pET-Blue-2 vector generating pET-*panD*. Recombinant *E. coli* BL21-DE3 harboring pETB-*panD* vector or vector control, induced with IPTG, were suspended in BugBuster reagent (Novagen, Madison, WI) 5 ml g^{-1} wet cells, for total soluble protein extraction. Benzonase (Novagen, Madison, WI) 1 μl ml^{-1}, β-mercaptoethanol 5 mM final concentration and a protease inhibitor cocktail as described (Rathinasabapathi *et al*., 2001) were added. Affinity purification of the recombinant protein was performed according to the manufacturer's instructions of ProBond resin (Invitrogen, Carlsbad, CA). The sample was further purified using a 5 ml DEAE-Sepharose ion-exchange column (Sigma, St. Louis, MO). Following protein elution using a 0–0.3 M linear NaCl gradient, the purified ADC-His was detected using SDS–PAGE gels. Total protein was estimated by the method of

Peterson (1977), and bovine serum albumin was the standard. The purified native ADC was used for raising polyclonal antibodies in rabbit according to manufacturer's protocol (Cocalico Biological, Reamstown, PA).

SDS–PAGE and immunoblot analyses

SDS–PAGE was performed 12% (w/v) Tris–glycine polyacrylamide gels (Sambrook *et al*., 1989). Protein samples were diluted with 2× of SDS–PAGE sample buffer containing 0.1 M Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 5 mM dithiotheritol, and 0.08% (w/v) bromophenol blue and denatured at 95 °C for 10 min. The separated proteins were visualized with Coomassie Brilliant Blue or silver stain. The SDS–PAGE separated proteins were transferred by electroblotting onto a PVDF membrane. The membranes were incubated with different dilutions of antibodies after blocking with blocking buffer [20 mM Tris–HCl, pH 7.5, 140 mM NaCl, and 5% (w/v) nonfat dry milk]. The primary anti-ADC polyclonal antibodies were used at 1:5000 dilution. The secondary anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used at a 1:30 000 dilution. After washing, binding of the antibody was recorded using a colorimetric substrate. The alkaline phosphatase activity was detected in 10 ml alkaline phosphatase buffer containing 0.1 M Tris–HCl pH 9, 0.1 M NaCl, 5 mM $MgCl_2$, 3.3 mg nitro blue tetrazolium and 1.7 mg bromochloroindolyl phosphate.

ADC activity assays

Aspartate decarboxylase was assayed using a radiometric procedure employing L-[U-^{14}C]aspartate. The assays contained in 50 mM potassium phosphate, pH 7.0, 1.2 mM of aspartate, 0.2 μCi of ^{14}C aspartate (217 mCi mmol^{-1}), 5 mM DTT, and crude protein or PEG (25%, w/v)-precipitated fraction in a total volume of 50 μl. Following incubation at 37 °C for 1 h, the reaction was terminated by adding 5 μl of 72% (w/v) trichloroacetic acid and the proteins were removed by centrifugation at 14 000 × g for 10 min. Radiolabeled $^{14}CO_2$ generated by ADC was trapped during the reaction with Whatmann no. 3 paper saturated with 20% (w/v) KOH. At the end of the reaction period the reaction products in the reaction mixture were separated from the substrate using thin layer chromatography using cellulose plate (Selecto Scientific, Georgia, USA) developed with solvents butanol:acetic acid:water (60:15:20, v/v/v), followed by autoradiography. The product formed was quantified by isolating the zone corresponding to β-ala. In a variation of the assay, $^{14}CO_2$ trapped from the assay was quantified in 50% (v/v) Ready Gel (Beckman Instruments, Fullerton, CA) in a Beckman liquid scintillation counter. The counting efficiency was 30%.

Germination tests

Seeds of *panD* homozygous transgenic lines, vector alone transgenic and wild-type control lines were surface sterilized. Around 100 seeds of each line were placed on 100 × 15 mm Petri plates containing half-strength MS salts. The plates were incubated in a growth chamber set at 24, 30, 36 or 42 °C under continuous light (20 μmol m^{-2} s^{-1}). Percent germination was scored over a period of 12 d based on radical emergence.

Growth tests and high temperature stress

For growth analysis and temperature stress, five-weeks-old seedling of two homozygous transgenic lines, vector control (R5) and WT control lines growing in 350 ml pots at 24 °C were used. The growing medium was Metro-Mix 300 (Scotts-Sierra, Marysville, OH) supplemented with 1 g slow-release fertilizer Osmocote (N:P:K 24:6:10, 5.8% ammoniacal N, 5% nitrate N, and 13.2% urea N) per pot. The pots were irrigated to container capacity every day. Seedlings were germinated and maintained in a culture room at 24 °C under 100 μmol m^{-2} s^{-1} light intensity 16 h light/8 h dark period for 5 weeks, then they were transferred to growth chambers adjusted at 24 or 35 °C with same light period and intensity. After 1 week, or 4 weeks, aboveground biomass, plant height, and number of leaves per plant were recorded.

Quantification of free β-ala, total free amino acids and pantothenate

Fully expanded leaf (1.0 g fresh wt.) was extracted using a methanol:chloroform:water mixture as described previously (Hanson and Gage, 1991).

The aqueous fraction was evaporated under a stream of N_2, redissolved in water and purified using a Dowex 50-H^+ ion exchange resin as described previously (Rhodes *et al*., 1989). Following pre-column derivatization of amino acids using phenylthiocyanate (PITC), the PTC-amino acid derivatives were separated and quantified by HPLC using Waters 515 HPLC Pump, Waters 717 plus Autosampler, Waters 2410 Refractive Index Detector, and YMC-Pack ODS-AM, S-5 μm, 12 nm 250×4.6 mm I.D. column, as described (Sherwood, 2001). Nor-leucine was used as the internal standard. Amino acid derivatives were identified at 254 nm based on retention times for pure standards run under identical conditions. Pantothenate was quantified in leaf extracts in 20 mM MOPS-KOH buffer pH 7.0, using a microbial assay with *Lactobacillus plantarum* (ATCC 8014) as described previously (Wyse *et al*., 1985).

Statistical treatment of data

All experiments were done at least twice with 3–6 replicates per treatment. The data were processed using the analysis of variance in a completely randomized design model using the SAS software (SAS, 2002). The mean separations were done using Duncan's multiple range test at $P \leq 0.05$ or 0.001.

Results

E. coli panD *gene is expressed in transgenic tobacco*

Tobacco was transformed with *Agrobacterium tumefaciens* strain ABI carrying (pMON-R5) vector or pMON-R5-*panD*, which contained *E. coli panD* gene under the control of CaMV 35S promoter and NOS3′ terminator. A total of 10 and 29 independent putative transformants were obtained for pMON-R5 and pMON-R5-*panD* constructs respectively based on their kanamycin resistance. In a PCR screen using genomic DNA template and primers specific for the *PanD* gene, all the *panD* putative transgenic plants amplified the expected 429 bp band which was not present in vector controls (Figure 1A).

Total RNA from 22 *panD* and 4 pMON-R5 transformants were analyzed in RNA blots, probed with [^{32}P]-labeled *panD* DNA. Fifteen *panD* transformants showed low, moderate or high levels of the expected *panD* transcript which was absent in the vector controls (pR1; Figure 1B). Some transformants with high level of *panD* transcript were analyzed by a Southern blot to determine the *panD* copy number. Genomic DNA, digested with *Hind*III, was separated on an agarose gel and blotted on nitrocellulose and probed with a *panD*-specific DNA probe. The *panD* gene sequence does not have a *Hind*III site but there is a *Hind*III site upstream of the CaMV 35S promoter. Eight *panD* transformants showed a single band, consistent with a single *panD* gene insertion (Figure 1C).

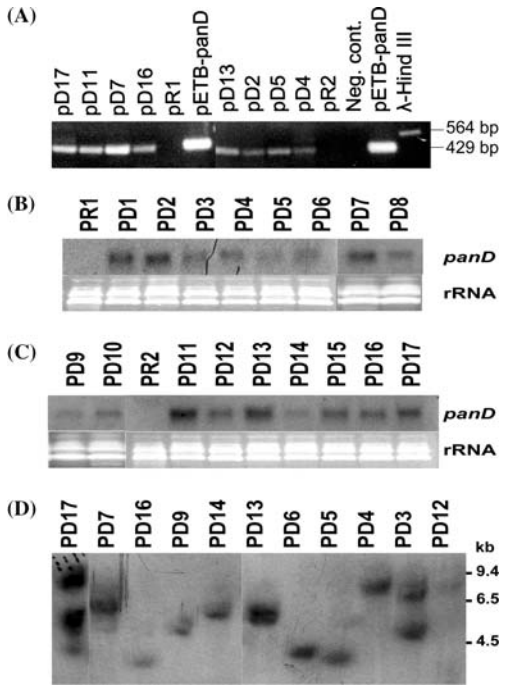

Figure 1. Transgene integration and RNA expression in primary transgenic tobacco. (A) PCR amplification of *panD* sequence from tobacco genomic DNA, pETB-*panD* is a positive control. (B) RNA blot probed with *panD* DNA probe (top) and EtBr stained RNA gel showing equal load (lower panel). (C) DNA blot probed with *panD* DNA probe. pR and pD lines are primary transgenic lines containing pMON-R5 and pMON-R5-*panD* respectively.

Some of the transformants with single *panD* gene per genome based on DNA blot analysis and positive for gene expression based on RNA blots were grown in a greenhouse and selfed. The progeny from these plants segregated 3:1 for kanamycin resistance: sensitivity in a seedling

bioassay, consistent with the single gene insertion (data not shown). Further analyses were done on two lines homozygous for the *panD* transgene (pD2 and pD7) and a vector control line (pR5) homozygous for the kanamycin resistance gene.

E. coli *ADC protein expressed in transgenic tobacco is active*

Protein extracts from the leaves of pD2 and pD7 showed detectable activities for ADC (Figure 2) and little activity was present in extracts from the pR5 transgenic line (Figure 2). The ADC activity in the PEG-concentrated crude protein extracted from transgenic lines pD2 and pD7 was 465 and 793 pmol mg^{-1} protein h^{-1} respectively. In immunoblots with ADC-specific polyclonal antibodies, a band corresponding to 13.8 kD, the unprocessed protein was revealed (Figure 2, inset). Both WT and pR5 plants did not show reaction with the ADC-specific polyclonal antibodies at 13.8 kD.

Free amino acid and pantothenate analyses in transgenic lines

Total free amino acids and β-ala levels in leaves were evaluated using HPLC separation of amino acid-PTC derivatives. Five-week-old plants were grown either at 24 or 35 °C for 1 week prior to analysis because of a thermotolerance phenotype of the *panD* lines (see below). At both the temperatures, 24 and 35 °C, pD2 and pD7 lines had higher levels of β-ala as a per cent of total amino acids compared to the pR5 and WT lines (Figure 3A and B). β-Ala was about 0.4% of the total free amino acids in the control lines and about 0.6% of the total free amino acids in the pD2 and pD7 lines (Figure 3A and B). Compared to the control lines, pD2 and pD7 had about 1.2–1.3× and 2–4× more absolute β-ala levels at 24 and 35 °C respectively (Figure 3A). The total free amino acid level in the *panD* lines was about 1.8–3.7× more than in control lines, at 35 °C but not at 24 °C (Figure 3B). An evaluation of pantothenate in fully expanded leaves from 4-month-old plants grown at 30 °C showed that pD2 and pD7 lines had about 3- to 4-fold higher levels of pantothenate than the control lines (Table 1).

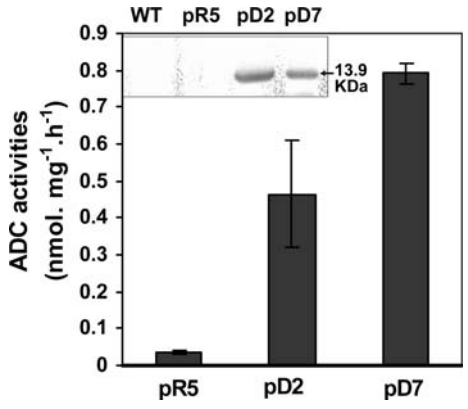

Figure 2. Expression of ADC protein in two transgenic lines homozygous for the *E. coli panD* gene. Specific activities of ADC in protein extracts. Inset: Western blot probed with *E. coli* ADC-specific polyclonal antibodies. WT = wild-type, pR5 = vector control, pD2 and pD7 = *panD* transgenic homozygous lines.

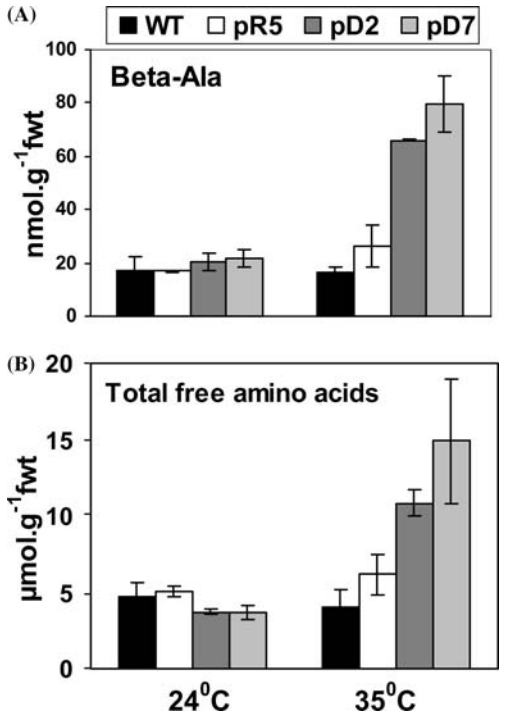

Figure 3. Transgenic tobacco lines expressing *E. coli panD* gene accumulate higher levels of β-ala and total free amino acids at 35 °C. (A) β-Ala; (B) total free amino acids levels. Fully expanded leaves were sampled from six-week-old seedlings either growing at 24 °C or after at 35 °C for 1 week prior to sampling. WT = wild-type, pR5 = vector control, pD2 and pD7 = *panD* transgenic homozygous lines Values are means and standard error for three independent analyses.

Table 1. Pantothenate levels in fully expanded leaves from 4-month-old plants grown at 30 °C.

Genotype	Pantothenate (nmol g^{-1} fwt)
WT	44.6 ± 1.5
pR5	42.6 ± 2.1
pD2	142.3 ± 26
pD7	174.9 ± 18

Values are means and standard errors for six replicate analyses.

Thermotolerance phenotype in ADC transgenic lines

To study the high temperature stress effect on transgenic lines, seeds were germinated and seedlings maintained at 24 °C for 5 weeks and then transferred to environmental growth chambers set at 35 °C. After 1 week of high temperature stress, WT and pR5 plant's height was reduced by 14.8% and 15.1% respectively compared to plants growing at 24 °C (Figure 4A). On the other hand, pD2 and pD7 lines gained 8% and 12% greater height at 35 °C compared to plants growing at 24 °C (Figure 4A). The pR5 and WT plants at 35 °C showed significant reduction in their leaf number compared to plants growing at 24 °C while pD2 and pD7 did not (Figure 4B). However, aerial biomass of the pD2 and pD7 lines did not differ significantly from those of WT line (Figure 4C). In another experiment, the plants were stressed for 1 week at 35 °C and then were allowed to recover for 3 weeks at 30 °C. The pD2 and pD7 lines gained significantly ($P \leq 0.001$) greater aerial biomass compared to WT and pR5 lines (Figure 5). The mean increases in fresh and dry weights were 54% and 84% respectively higher than that achieved by the pR5 and WT plants during recovery from high temperature stress (Figure 5).

Transgenic expression of ADC improves tobacco seed germination at 42 °C

When seeds of *panD* lines pD, vector control pR and wild-type seeds were germinated at 30 °C, all the lines germinated equally well (data not shown). However, when the imbibed seeds were incubated at 42 °C, lines expressing the *panD* gene germinated better than the vector controls (Figure 6A). Three out of five *panD* lines germinated better than the wild-type also (Figure 6A). A time course

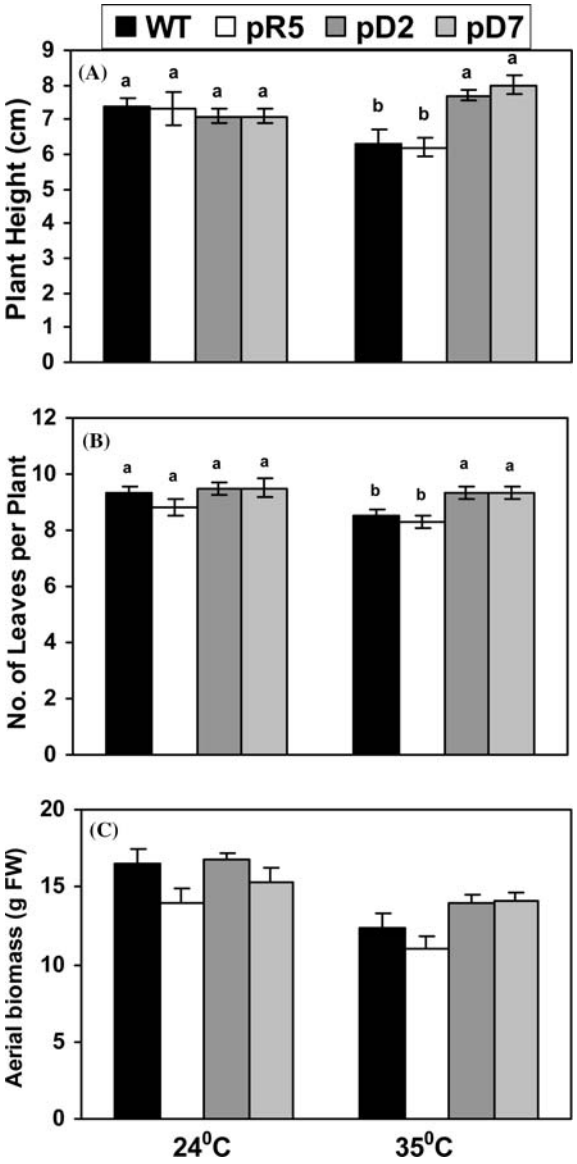

Figure 4. Expression of *E. coli panD* gene in transgenic tobacco improves growth and biomass under high temperature. Five-week-old seedlings from wild-type, pR5, pD2 and pD7 lines were grown at 24 and 35 °C for 1 week and harvested for plant height (A), number of leaves per plant (B), and aerial biomass (C). WT = wild-type, pR5 = vector control, pD2 and pD7 = *panD* transgenic homozygous lines. Data are means (± S.E.) from six plants.

experiment at 42 °C showed that pD2 and pD7 lines had significantly higher ($P < 0.05$) germination rate than the vector control and wild-type (Figure 6B). Vector control line had significantly lower ($P < 0.05$) germination rate than the wild-type at 42 °C (Figure 6B).

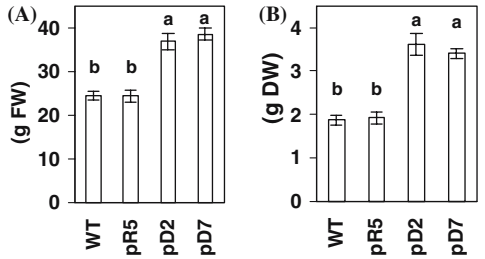

Figure 5. Expression of *E. coli panD* gene in transgenic tobacco improves plants' recovery from heat stress. Five-week-old seedlings from wild-type, pR5, pD2 and pD7 lines were stressed for 1 week at 35 °C and then allowed to recover for 3 weeks at 30 °C and harvested for aerial biomass fresh weight (A) and dry weight (B). WT = wild-type, pR5 = vector control, pD2 and pD7 = *panD* transgenic homozygous lines. Data are means (±S.E.) from six plants.

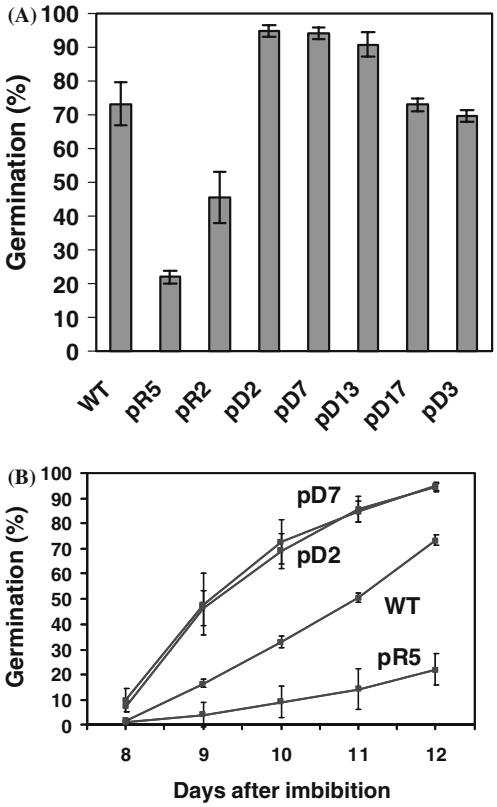

Figure 6. Expression of *E. coli panD* gene in transgenic tobacco improves germination rate and percent germination at 42 °C. (A) Germination percent at 42 °C at day 12 after sowing. (B) Germination percent at 42 °C scored over 12 d after sowing. WT, pR vector control, = *panD* transgenic lines. Data are means (±S.E.) from six independent Petri dishes.

Discussion

Despite extensive characterizations of *Escherichia coli panD* gene and its product, the *panD* gene was not previously expressed in an eukaryotic host. Here, the *E. coli panD* gene was expressed in transgenic tobacco under the control of a constitutive promoter. The gene was integrated in the tobacco genome (Figure 1A and C), the transcriptional level of *panD* gene varied within primary transformants (Figure 1B) and the transgene was stably inherited.

The bacterial pyruvoyl-dependent ADC enzyme was active in the transgenic tobacco (Figure 2). The ADC is known to be translated as a proenzyme that undergoes self-processing at a Gly_{24}–Ser_{25} bond to produce a β-subunit (2.8 kD) and an α-subunit (11.0 kD) with a pyruvoyl group at its N-terminus, derived from serine (Ramjee *et al.*, 1997). This processing is essential for generating a pyruvoyl as a catalytically active prosthetic group and hence enzyme activity (Ramjee *et al.*, 1997). Although, ADC is unique to prokaryotes and not found in eukaryotes (Rathinasabapathi *et al.*, 2000; White *et al.*, 2001), we expected that it will be correctly processed in its plant host since there are other pyruvoyl-dependent enzymes requiring similar post-translational modifications in plants such as s-adenosyl-L-methionine decarboxylase and phosphatidylserine decarboxylase (Xiong *et al.*, 1997; Rontein *et al.*, 2003). The protein blot analysis for tobacco crude protein using ADC-specific antibodies labeled a band corresponding to the unprocessed π-peptide (Figure 2, inset). The relative intensities of the unprocessed π-peptide in the immunoblot was consistent with the enzyme activities of the two transgenic lines. The pD7 line with less relative intensities of π-peptide, and hence more processed protein, had higher relative enzymatic activities compared to the pD2 line. However, the blot analyses were not sensitive enough to detect the processed α- and β-subunits. This could be because of incomplete processing of the proenzyme, resulting in low abundance of α- and β-subunits. We found similar phenomenon with *E. coli* crude protein (Fouad, 2004).

When leaf free amino acids were analyzed, there was a significant increase in β-ala levels up to 3.7-fold in the *panD* transgenic lines compared to control lines growing at the same conditions (Figure 3). There was also an increase in the total

free amino acids in the *panD* lines at 35 °C (Figure 3). While wild-type and vector control transgenic plants synthesize their β-ala through the endogenous pathway, the *panD* transgenic lines had elevated levels of β-ala due to L-aspartate decarboxylation. ADC is not subject to feedback inhibition by β-ala, pantothenate, coenzyme A or acetyl coenzyme A (Williamson and Brown, 1979; Cronan, 1980). This is consistent with the increase in β-ala level in the *panD* transgenics. We expected that expression of bacterial ADC in tobacco would not negatively affect the aspartate pool since other work showed that the aspartate pool in plants is flexible for metabolic engineering (Galili and Hoefgen, 2002). Although many studies have been done on free amino acid levels in plants, only a rare few quantified non-protein amino acids. Here, we quantified β-ala using HPLC separation of PTC-derivatized amino acids.

The *panD* transgenic lines also contained significantly higher levels of pantothenate than the control lines (Table 1). Pantothenate is synthesized by the condensation of β-alanine with D-pantoate, which is derived from valine (Raman and Rathinasabapathi, 2004). Expressing *Corynebacterium glutamicum panD* gene in *E. coli* resulted in elevated level of both β-ala and pantothenate (Dusch *et al.*, 1999), suggesting that β-ala availability was limiting pantothenate synthesis in bacteria. Future experiments are needed to test whether β-ala is rate-limiting for pantothenate synthesis in plants and whether the *panD* lines also have increased levels of valine, the precursor for D-pantoate.

When the plants were exposed to high temperature stress (35 °C), transgenic lines expressing bacterial ADC, showed less inhibition by stress (Figure 4). In a high temperature stress recovery experiment, the *panD* transgenic lines recovered significantly better than the wild-type and vector control lines (Figure 5A and B). Thermotolerance phenotype could also be found in germinating seeds. Seeds of *panD* lines had an increased germination rate and germination percent at 42 °C compared to the vector control and wild-type lines (Figure 6). This suggests that the transgene is expressed from the earliest stages of plant development and improves thermotolerance. These findings are consistent with the properties known for bacterial ADC. Self-processing and specific activity of bacterial ADC were promoted *in vitro* by a high temperature treatment (Ramjee *et al.*, 1997; Chopra, *et al.*, 2002). Therefore, it is conceivable that ADC was self-processed and has better activity at high temperature and this therefore, may have resulted in the increased biomass phenotype under high temperature.

Increased thermotolerance trait will be valuable for crop improvement. Although the mechanisms by which L-aspartate α-decarboxylation interacts with plant metabolism are not clear, we speculate that this could be mediated *via* the products of ADC reaction, namely β-ala, CO_2 or both.

Firstly, β-ala could have biological activities when accumulated in plant cells, such as acting as chaperones interacting with heat shock proteins, membranes and enzymes (Mehta and Seidler, 2005). Consistent with this, the increased level of β-ala in *panD* transgenic lines could have provided thermotolerance at seed germination and whole plant stage. Other work using cultured plant cells or whole plants showed that endogenous β-ala levels increased under heat stress (Mayer *et al.*, 1990; Kaplan *et al.*, 2004) or drought stress (Rizhsky *et al.*, 2004). A similar role can also be speculated for pantothenate.

Secondly, β-ala could work as a signaling molecule, turning on pathways that improved plant growth, similar to GABA (Bouche and Fromm, 2004), a four carbon analog of β-ala. The elevated level of β-ala may increase the flux toward L-aspartate biosynthesis which in turn could have improved nitrogen utilization and reduced C/N ratio. Although other metabolic engineering efforts in amino acid metabolism such as over expressing glutamine synthetase and glutamate synthase resulted in improved total free amino acids and growth phenotypes in transgenic plants (Ameziane *et al.*, 2000; Migge *et al.*, 2000; Chichkova *et al.*, 2001; Fuentes *et al.*, 2001; Oliveira *et al.*, 2002; Fu *et al.*, 2003), the bacterial L-aspartate-α-decarboxylase was not previously expressed in transgenic plants. Together, the data reported here clearly show that total free amino acids, β-ala and pantothenate levels, and thermotolerance can be improved in plants by simply expressing a bacterial gene involved in nitrogen metabolism.

Thirdly, the elevated level of β-ala could have affected the endogenous β-ala biosynthetic pathway(s) by feedback inhibition. This in return could have provided extra β-ala-precursors, such as uracil and polyamines (Raman and Rathinasabapathi,

2004), to be available for other biologically significant use.

Finally, ADC-generated CO_2 can be transferred to the chloroplast and be available for fixation by Rubisco. Such increase in endogenous [CO_2] could result in improving the carboxylation activity of the Rubisco and leaf-water status and also reducing the photorespiration (Long *et al.*, 2004). Since the ADC activities found in the *panD* transgenics are relatively low (Figure 2), this increase can only be expected to be marginal. However, such low increases in [CO_2] could have had an effect stimulating nitrogen or carbon assimilation (Stitt and Krap, 1999) and improving stress adaptation (Taub *et al.*, 2000; Wullschleger *et al.*, 2002).

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