

Plant C-N Hydrolases and the Identification of a Plant N-Carbamoylputrescine Amidohydrolase Involved in Polyamine Biosynthesis*

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A nitrilase-like protein from *Arabidopsis thaliana* (NLP1) was expressed in *Escherichia coli* as a His₆-tagged protein and purified to apparent homogeneity by Ni²⁺-chelate affinity chromatography. The purified enzyme showed N-carbamoylputrescine amidohydrolase activity, an enzyme involved in the biosynthesis of polyamines in plants and bacteria. N-carbamoylputrescine amidohydrolase activity was confirmed by identification of two of the three occurring products, namely putrescine and ammonia. In contrast, no enzymatic activity could be detected when applying various compounds including nitriles, amines, and amides as well as other N-carbamoyl compounds, indicating the specificity of the enzyme for N-carbamoylputrescine. Like the homologous β -alanine synthases, NLP1 showed positive cooperativity toward its substrate. The native enzyme had a molecular mass of 279 kDa as shown by blue-native polyacrylamide gel electrophoresis, indicating a complex of eight monomers. Expression of the NLP1 gene was found in all organs investigated, but it was not induced upon osmotic stress, which is known to induce biosynthesis of putrescine. This is the first report of cloning and expression of a plant N-carbamoylputrescine amidohydrolase and the first time that N-carbamoylputrescine amidohydrolase activity of a recombinant protein could be shown *in vitro*. NLP1 is one of the two missing links in the arginine decarboxylase pathway of putrescine biosynthesis in higher plants.

The term “C-N Hydrolases” was introduced by Bork and Koonin in 1994 (1) describing a protein superfamily whose members are all involved in the cleavage of C-N bonds but display only moderate homology (12–24% amino acid identity). Members of this family are found from bacteria to man and can be classified into 13 branches (2, 3). The family includes the following: (i) nitrilases (and cyanide hydratases) and aliphatic amidases, which hydrolyze nitriles (–CN) or amides (–CONH₂), respectively, to the corresponding carboxylic acids; (ii) β -alanine synthases (also called β -ureidopropionases), which produce β -alanine from N-carbamoyl- β -alanine (β -ureidopropionic acid); and (iii) other amidohydrolases whose natural substrates are largely unknown. The genome of *Arabidopsis*

thaliana encodes eight C-N hydrolases: (i) four nitrilases, one of which (NIT4) was identified as β -cyanoalanine hydratase/nitrilase (4); (ii) one β -alanine synthase involved in pyrimidine catabolism (5); and (iii) three other nitrilase-like proteins (NLPs)¹ whose function is yet unknown. One of these, provisionally called NLP1, shows high homology to a recently characterized protein of *Pseudomonas aeruginosa*, the function of which was defined as N-carbamoylputrescine amidohydrolase, involved in polyamine biosynthesis in this species (6).

Polyamines are low molecular mass polycations of vital function for all living organisms. A key intermediate in polyamine biosynthesis is putrescine (H₂N(CH₂)₄NH₂), which is already a polyamine and is further converted to higher polyamines like spermidine and spermine. In principle, two different biosynthetic pathways lead to the formation of putrescine: (i) the ornithine decarboxylase (ODC) pathway through the decarboxylation of ornithine by ODC; and (ii) the arginine decarboxylase (ADC) pathway through the successive reactions of arginine decarboxylase (producing agmatine from arginine), agmatine iminohydrolase (producing N-carbamoylputrescine from agmatine), and finally N-carbamoylputrescine amidohydrolase (producing putrescine from N-carbamoylputrescine). Both pathways are described as occurring in plants (reviewed in Ref. 7), but recently it was shown that in *A. thaliana* no ODC gene exists and that the ornithine decarboxylating activity found in extracts of this plant was non-enzymatic (8). This stresses the importance of the ADC-pathway for plants, at least with respect to *A. thaliana*. Many genes of plant polyamine biosynthesis have been cloned (for review see Ref. 9), but until now the genes of the ADC-pathway encoding agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase were not known. We will show here that the C-N hydrolase NLP1 of *A. thaliana* encodes a functional N-carbamoylputrescine amidohydrolase.

EXPERIMENTAL PROCEDURES

Synthesis of N-Carbamoylputrescine—Synthesis of N-carbamoylputrescine-HCl was done according to Smith and Garraway (10). Purification of N-carbamoylputrescine was performed as follows. After removal of the side product N,N-dicarbamoylputrescine by filtration, the filtrate was evaporated to dryness. The residue was extracted with ethanol, and the extract again was evaporated to dryness. The residue was then dissolved in 50 ml of water, neutralized using 2 M HCl, and loaded on a column (25 cm \times 1.5 cm) of Dowex 50 X 8 (Li-form). Elution was done with 3-aminopropyl-1,3-diaminopropane (0.2 M, neutralized with 2 M HCl). The eluting fractions were tested by thin layer chromatography for ninhydrin-positive substances. Fractions showing only one ninhydrin-positive spot were combined, evaporated to dryness, and

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¹ The abbreviations used are: NLP, nitrilase-like protein; ADC, arginine decarboxylase; CPA, N-carbamoylputrescine amidohydrolase; ESI-TOF, electrospray ionization-time-of-flight; EST, expressed sequence tag; ODC, ornithine decarboxylase.

recrystallized from 96% (v/v) ethanol. The final product (165 mg) showed a melting point of 186–187 °C (reported melting point of N-carbamoylputrescine, 185–186 °C).

Cloning and Expression of NLP1—NLP1 was received as an EST clone (70G11T7, GenBank™ accession number AA586100) from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) and cloned into the pET-21b(+)-vector (Novagen, Madison, WI) using the same strategy as described for the four nitrilases of *A. thaliana* (11). In brief, the NLP1 open reading frame was amplified by PCR using the EST plasmid DNA as a template, simultaneously introducing an *Nde*I restriction site (including the start codon CATATG) at the 5'-end and a *Sal*I restriction site instead of the stop codon at the 3'-end. The PCR product was then cloned into the *Nde*I and *Xho*I sites of the pET-21b(+)-vector. Expression was done in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). After purification of the His₆-tagged NLP1 using Ni²⁺-chelate chromatography (Qiagen, Hilden, Germany), the purified protein was desalted using a PD-10 column (Amersham Biosciences), which was equilibrated in 50 mM potassium phosphate, pH 8.0, and 1 mM dithiothreitol. Protein concentrations were between 8 and 12 µg/µl. The purified protein was shock frozen in liquid nitrogen and stored at -80 °C.

Activity Measurements—The standard reaction contained 1 µg of protein in 50 mM potassium phosphate, 1 mM dithiothreitol, pH 8.0, in a final volume of 1 ml. Substrates were added at a final concentration of 1–3 mM. Reactions were carried out for 1–2 h at 37 °C or, for determination of the kinetic parameters of NLP1, for 30 min at 30 °C. Ammonia production was measured using the Berthelot reaction as described elsewhere (4). Production of putrescine was shown qualitatively and semiquantitatively by thin layer chromatography. Aliquots (5 µl) of the sample were spotted on silica plates (Polygram SIL G/UV₂₅₄, Macherey-Nagel, Düren, Germany), which were subsequently developed in ethanol/NH₄OH (25% (v/v)) 1:1. N-Carbamoylputrescine and putrescine were visualized with ninhydrin.

Mass Spectrometric Analysis of the Reaction Product—A standard reaction as described above using N-carbamoylputrescine as the substrate was performed with the modification that the buffer concentration was reduced to 5 mM. After 2 h, a 100-µl aliquot was withdrawn, and 400 µl of ethanol were added. After boiling for 10 min, the denatured protein was removed by centrifugation (15 min at 13,000 rpm in a tabletop centrifuge), and the supernatant evaporated to dryness. The residue was resuspended in 500 µl of 0.5 mM pentadecafluorooctanoic acid, and insoluble material was precipitated by centrifugation (15 min, 13,000 rpm). The soluble supernatant was diluted 10-fold in 0.5 mM pentadecafluorooctanoic acid and infused at 5 µl/min into a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) with the following settings: capillary, 3000 V; cone voltage, 25 V; collision energy, 5 eV; collision gas off.

Blue-native Polyacrylamide Gel Electrophoresis—Blue-native polyacrylamide gel electrophoresis was performed as described by Schägger *et al.* (12) for soluble proteins using a 4–15% (w/v) separating gel.

Construction of Phylogenetic Tree—Fifty-five amino acid sequences of C-N hydrolases, therein 31 nitrilases (including two cyanide-hydratases), three aliphatic amidases, four β -alanine synthases (β -ureidopropionases), one N-carbamoyl-D-amino acid amidohydrolase, one putative N-carbamoylputrescine amidohydrolase, and 15 C-N hydrolases of yet unknown substrate-specificities were aligned using the DNAMAN software (version 5.2.2, Lynnon Biosoft, Vaudreuil, Quebec, Canada), and a phylogenetic tree was constructed using the same software with a maximum likelihood algorithm and 100 bootstrap trials.

Northern Blot Analysis—Leaves (~1 g) were incubated on a potassium phosphate buffer (50 mM, pH 5.8) or on a buffer that included sorbitol (0.4 M) for 12 h under constant illumination. Extraction of RNA, formaldehyde-agarose gel electrophoresis, blotting on Hybond-N+ nylon membranes (Amersham Biosciences), and hybridization to radiolabeled probes was done following standard protocols (13). DNA fragments of the arginine decarboxylase genes 1 and 2 were amplified from genomic DNA by PCR using the sequences published in GenBank™ (accession numbers U52851 and AF009647, respectively) as described (14). Radiolabeled probes were synthesized using the High Prime DNA labeling kit (Roche Diagnostics) and [α -³²P]dATP (110 TBq mmol⁻¹, Amersham Biosciences).

RESULTS AND DISCUSSION

Identification of Arabidopsis NLPs—In the course of our studies on plant nitrilases (4, 11) we detected genes from *A. thaliana* coding for proteins homologous to nitrilases, which we

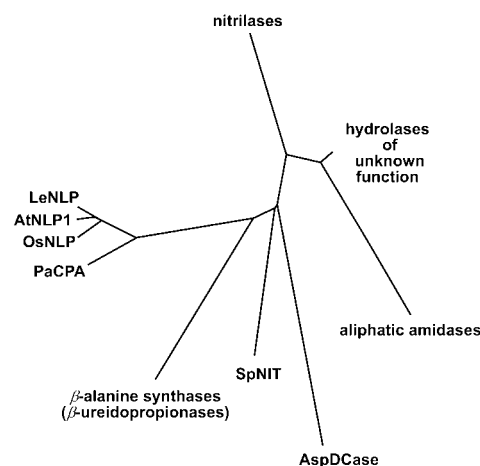


FIG. 1. Simplified phylogenetic tree of C-N hydrolases. For the sake of clarity, only the main lineages as well as the branches relevant for this work are shown. For details of construction of the tree, see "Experimental Procedures." AspDCase, N-carbamoyl-D-amino acid amidohydrolase of *Agrobacterium* sp. (20); AtNLP1, nitrilase-like protein of *A. thaliana* (see "Results and Discussion"); LeNLP, nitrilase-like protein of *Lycopersicon esculentum* (GenBank™ accession number Y19104); OsNLP, nitrilase-like protein of *Oryza sativa* (GenBank™ accession number AB062681); PaCPA, N-carbamoylputrescine amidohydrolase of *P. aeruginosa* (6); SpNIT, putative nitrilase of *Schizosaccharomyces pombe* (GenBank™ accession number AL023590).

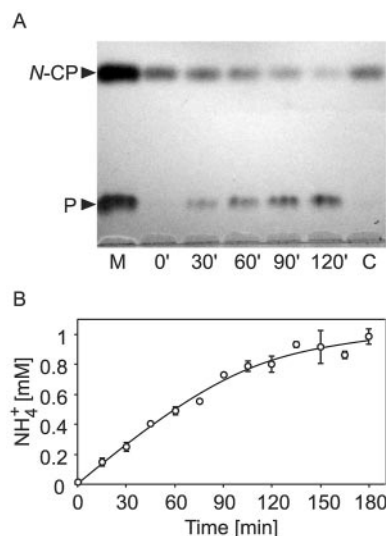


FIG. 2. Hydrolysis of N-carbamoylputrescine to putrescine and ammonia by NLP1. A, NLP1 was incubated with N-carbamoylputrescine (1 mM) as described under "Experimental Procedures." After the indicated times, aliquots were withdrawn and subjected to thin layer chromatography. The positions of N-carbamoylputrescine (N-CP) and putrescine (P) are indicated by arrowheads. B, production of ammonia as measured by the indophenol blue method. M, mix of N-carbamoylputrescine and putrescine; C, heat-denatured control after 2 h of reaction.

subsequently called provisionally nitrilase-like proteins.² Of the three NLPs existing in *A. thaliana* (NLP1, gene At2g27450; NLP2, gene At4g08790; and NLP3, gene At5g12040), NLP1 is annotated as a putative nitrilase and shows high homology to an EST clone from tomato, which is annotated as β -alanine synthase (88% identity, GenBank™ accession number Y19104). However, NLP1 showed no β -alanine synthase activity² (*vide infra*), and recently the real β -alanine-synthase from *A. thaliana* was cloned (5). Phylogenetic analysis of a large

² M. Piotrowski, unpublished results.

number of C-N hydrolases revealed that NLP1, together with the above mentioned C-N hydrolase from tomato, lies on one branch with β -alanine synthases and an *N*-carbamoyl-D-amino acid amidohydrolase from *Agrobacterium* sp. (*AspDCase* in Fig. 1). These enzymes have in common the fact that they hydrolyze *N*-carbamoyl compounds, and it could therefore be speculated that the natural substrate for NLP1 and its tomato homolog is also an *N*-carbamoyl compound. Recently, the gene of a *N*-carbamoylputrescine amidohydrolase from *P. aeruginosa* was identified (6) (*PaCPA* in Fig. 1), and the coded amino acid sequence shows high similarity to NLP1 (67% identity). This is not only in line with the above mentioned hypothesis but moreover points to *N*-carbamoylputrescine as a possible substrate of NLP1. *PaCPA* belongs to the same family (family 11) of C-N hydrolases (as classified by Pace and Brenner, Refs. 2 and 3) as NLP1. The function of the members of this family was formerly unknown, but it now seems to emerge that they are involved in polyamine biosynthesis (Ref. 6, and *vide infra*).

NLP1 Is a Functional *N*-Carbamoylputrescine Amidohydrolase—The cDNA of NLP1 was obtained from the Arabidopsis Biological Resource Center as an EST clone and was subse-

quently sequenced. It contains a proposed complete open reading frame of 900-bp coding for 299 amino acids and 31 bp of the 5'-UTR. The open reading frame, which is 100% identical to the published sequence derived from the genome data (GenBankTM accession number NM_128305) was amplified by PCR and cloned into the pET-21b(+)-vector. NLP1 was expressed as His₆-tagged protein in *E. coli* and purified using Ni²⁺-chelate affinity chromatography to apparent homogeneity as judged by Coomassie-stained SDS-polyacrylamide gels. The purified enzyme displayed *N*-carbamoylputrescine activity as shown by different methods as follows. (i) By thin layer chromatography it could be shown that *N*-carbamoylputrescine vanished during the time course of the reaction while a product, showing the same *R_f* value as authentic putrescine, was formed (Fig. 2A). (ii) This product could be identified as putrescine by mass spectrometric analysis, because it showed the same ESI-TOF spectrum as authentic putrescine (Fig. 3). (iii) In addition to putrescine, NH₄⁺ was formed as shown by the indophenol blue method (Fig. 2B).

NLP1 showed no activity toward the *N*-carbamoyl compounds *N*-carbamoyl- β -alanine (β -ureidopropionic acid) and *N*-carbamoyl-D,L-aspartic acid as judged by thin layer chromatography (data not shown) and the measurement of liberated NH₄⁺ (Table I). Of some other substances tested, including nitriles, amides, and intermediates of polyamine biosynthesis, none were substrates of NLP1 (Table I).

This is the first time that cloning of an *in vitro* active *N*-carbamoylputrescine amidohydrolase is reported. This enzyme is of vital importance for *A. thaliana*, because putrescine bio-

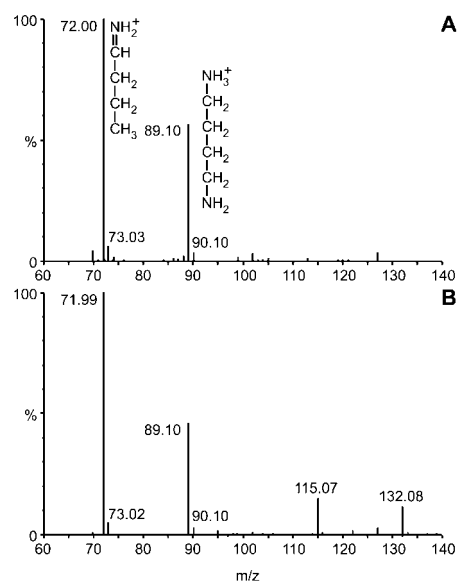


FIG. 3. Mass spectrometric identification of the reaction product as putrescine. A, ESI-TOF spectrum of authentic putrescine. The quasi-molecular ion ($[M+H]^+$, m/z 89.1) as well as a fragment of putrescine (m/z 72.0) is visible. B, ESI-TOF spectrum of an aliquot of the same reaction as shown in Fig. 2A. The signals at m/z 132.1 and m/z 115.1 are derived from residual *N*-carbamoylputrescine (quasi-molecular ion and fragment, respectively).

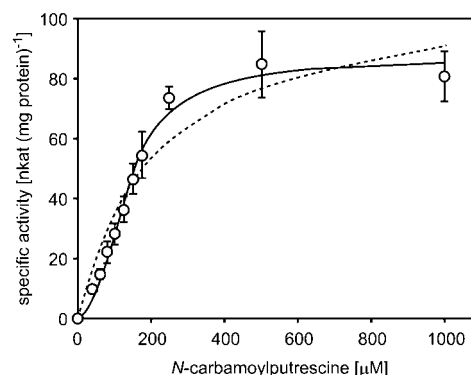


FIG. 4. Dependence of velocity on substrate concentration. NLP1 was incubated with *N*-carbamoylputrescine at the indicated concentrations. The curve was calculated by non-linear regression to the Hill equation = $V_{max} \cdot [S]^h / (K + [S]^h)$. The dotted line represents the non-linear regression to the Michaelis-Menten equation $V = (V_{max} \cdot [S]) / (K_m + [S])$.

TABLE I
Substrate specificity of NLP1

Purified NLP1 (1 μ g) was incubated with the given substrates at a final concentration of 3 mM in a total volume of 1 ml for 4 h at 37 °C. Activity was measured as ammonia-producing activity as described under "Experimental Procedures." N.D., not detectable.

Substrate	Relative activity	Typical substrate for
	%	
<i>N</i> -Carbamoylputrescine	100	
<i>N</i> -Carbamoyl-D,L-aspartic acid	N.D.	<i>Agrobacterium</i> sp. DCase
<i>N</i> -Carbamoyl- β -alanine	N.D.	β -Alanine synthase
3-Phenylpropionitrile	N.D.	<i>A. thaliana</i> NIT1
Allylcyanide	N.D.	<i>A. thaliana</i> NIT1
β -Cyano-L-alanine	N.D.	<i>A. thaliana</i> NIT4
Indolyl-3-acetonitrile	N.D.	<i>A. thaliana</i> NIT1
Agmatine	N.D.	Agmatine iminohydrolase
L-Asparagine	N.D.	Asparaginase
L-Glutamine	N.D.	Glutaminase
L-Arginine	N.D.	Arginine deiminase
L-Citrulline	N.D.	Citrullinase

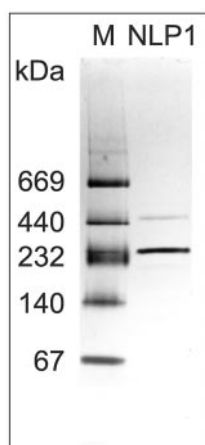


FIG. 5. **Native molecular mass of NLP1.** The native molecular mass of NLP1 was determined by blue-native polyacrylamide gel electrophoresis. Approximately 1 μ g of protein was separated on a 4–15% (w/v) gradient separating gel. *M*, native high molecular mass marker.

synthesis via the ODC pathway (*vide supra*) does not occur in *A. thaliana* because of the absence of ornithine decarboxylase (8). Using BLAST search (15) sequences for homologues of NLP1 can be found for many plant species, indicating that the ADC pathway of putrescine biosynthesis may be widespread among higher plants (data not shown). To completely uncover the putrescine biosynthesis pathway of *A. thaliana*, cloning a functional agmatine iminohydrolase (agmatine deiminase) is necessary.

Biochemical Characterization of NLP1—NLP1 showed maximal activity at $\sim 40^\circ\text{C}$ and between pH 8 and 9. Determination of kinetic parameters revealed that NLP1 showed no Michaelis-Menten kinetics toward *N*-carbamoylputrescine (Fig. 4, dotted line). The v/s graph was found to be sigmoid and could be described by the Hill equation $v = (V_{\max}[S]^h)/(K + [S]^h)$. Non-linear regression of the data to this equation (Fig. 4, solid line) gave a Hill coefficient (h) of 2.2, indicating positive cooperative binding of the substrate, and a V_{\max} value of 86 nanokatals (mg of protein) $^{-1}$. Half maximal velocity was reached at 135 μM substrate. Cooperative binding of the substrate with comparable h values was also found for β -alanine synthases of man and rat (16, 17), enzymes showing high similarity to NLP1 (see Fig. 1). A reason for cooperative substrate binding may be allosterism of the enzyme. Most C-N hydrolases studied so far are forming homomultimers ranging from tetramers to hexadecamers. We tested the multimeric structure of NLP1 using blue-native polyacrylamide gel electrophoresis (Fig. 5). NLP1 showed a native molecular mass of 279 kDa, indicating that it consists of eight monomers. A fainter band corresponding to a molecular mass of 432 kDa, indicative of a dodecamer, could not be detected in all preparations and may be a contaminating protein of *E. coli*. Further studies regarding allosteric behavior of NLP1 are currently underway.

Expression of NLP1—We investigated the expression of the *NLP1* gene in comparison to the two arginine decarboxylase genes of *A. thaliana* (*ADC1* and *ADC2*, Ref. 18) in different organs by Northern-blot analysis (Fig. 6A). Flowering plants were divided into roots, stems, rosette leaves, and complete flowers. In all organs investigated, the mRNA of *ADC1*, *ADC2*, and *NLP1* was detectable. The highest expression for all three genes was found in the roots and the flowers, whereas expression in stems and leaves was lower. These results are in contrast to those shown by Soyka and Heyer (14), who found only weak expression of *ADC1* and *ADC2* in roots and a strong expression of *ADC1* in rosette leaves. However, the stronger

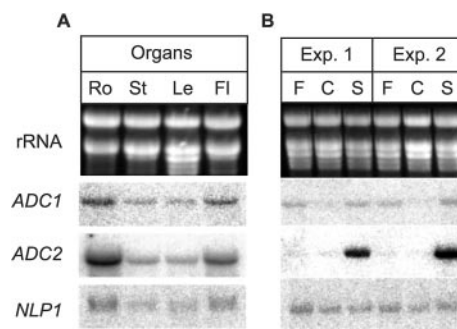


FIG. 6. **Expression of ADC pathway genes in different organs of *A. thaliana* (A) and during osmotic stress (B).** For panel B, leaves (~ 1 g) were incubated on 50 mM potassium phosphate (pH 5.8) (C) or 50 mM potassium phosphate (pH 5.8) plus 0.4 M sorbitol (S) for 12 h or harvested immediately prior to RNA-extraction (F, fresh). Ten μ g of total RNA each were separated by agarose-gel electrophoresis and blotted onto a nylon membrane. Hybridization was done with radioactive-labeled probes against the *ADC* genes and the *NLP1* gene. *Ro*, root; *St*, stem; *Le*, leaf; *Fl*, flower.

expression of ADC pathway genes in roots compared with rosette leaves and stems shown here is in agreement with the ~ 2 -fold higher putrescine levels of roots compared with shoots reported by Watson *et al.* (18). It is also worth mentioning that most of the *A. thaliana* EST clones coding for NLP1 were made from root mRNA. We next investigated the expression of *NLP1* in leaves under osmotic stress, which leads to a strong increase of putrescine levels in *A. thaliana*, concomitant with an increase of ADC activity caused by a strong induction of the *ADC2* gene (14, 19). As expected, we could show a strong induction of the *ADC2* gene upon osmotic stress treatment (Fig. 6B), whereas the expression of *ADC1* was very low in buffer-treated leaves and reached comparable levels in stressed leaves and freshly harvested leaves. The expression of *NLP1* kept constant under the conditions tested, indicating that *N*-carbamoylputrescine amidohydrolase is not the rate-limiting enzyme in osmotic stress-induced putrescine biosynthesis. The expression of *NLP1* was found to be very low; we did not detect the protein in crude extracts of leaves by use of an α -NLP1 antibody, nor could we show *N*-carbamoylputrescine amidohydrolase activity using the NH_3 assay (data not shown).

Name for NLP1—"NLP1" was only a provisional name based on the homology of this enzyme to nitrilases. Because enzyme names defined by function are preferable, we propose to call this enzyme CPA (for *N*-carbamoylputrescine amidohydrolase). We could also show that the tomato NLP, which was annotated as β -alanine-synthase, is a functional CPA (data not shown).

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