The Arabidopsis thaliana Isogene NIT4 and Its Orthologs in Tobacco Encode β-Cyano-L-alanine Hydratase/Nitrilase*

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Nitrilases (nitrile aminohydrolases, EC 3.5.5.1) are enzymes that catalyze the hydrolysis of nitriles to the corresponding carbon acids. Among the four known nitrilases of Arabidopsis thaliana, the isoform NIT4 is the most divergent one, and homologs of NIT4 are also known from species not belonging to the Brassicaceae like Nicotiana tabacum and Oryza sativa. We expressed A. thaliana NIT4 as hexahistidine tag fusion protein in Escherichia coli. The purified enzyme showed a strong substrate specificity for β-cyano-L-alanine (Ala(CN)), an intermediate product of cyanide detoxification in higher plants. Interestingly, not only aspartic acid but also asparagine were identified as products of NIT4-catalyzed Ala(CN) hydrolysis. Asn itself was no substrate for NIT4, indicating that it is not an intermediate but one of two reaction products. NIT4 therefore has both nitrilase and nitrile hydratase activity. Several lines of evidence indicate that the catalytic center for both reactions is the same. The NIT4 homologs of higher plants. Interestingly, not only aspartic acid but also asparagine were identified as products of NIT4-catalyzed Ala(CN) hydrolysis. Asn itself was no substrate for NIT4, indicating that it is not an intermediate but one of two reaction products. NIT6 therefore has both nitrilase and nitrile hydratase activity. Several lines of evidence indicate that the catalytic center for both reactions is the same. The NIT6 homologs of higher plants have a quite different substrate specificity compared with the NIT1/NIT2/NIT3 group. Here, we report about the elucidation of the enzymatic function of the NIT4 enzyme family.

EXPERIMENTAL PROCEDURES

Plant Material—A. thaliana ecotype C24 and Nicotiana tabacum W38 were grown in a greenhouse in standard soil at 20 °C, 70% relative humidity, and 210 μmol photons m⁻² s⁻¹ for a 16-h photoperiod. Seeds of L. angustifolius were sown on Vermiculite and grown in a growth chamber under the following climatic conditions: 16-h photoperiod, 120 μmol photons m⁻² s⁻¹, 24 °C during photoperiod, 20 °C during night, 70% relative humidity.

General Procedures—The following general procedures have been described elsewhere (4): sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein determination.

Vector Construction and Cloning of Nitrilase 4 cDNAs—All basic molecular techniques were adapted from Ausubel et al. (12) or Sam-brook et al. (13). Sequences of polymerase chain reaction-amplified or mutated cDNAs were verified by sequencing. Cloning of NIT4 cDNA was described previously (4). The cDNAs for the N. tabacum nitrilases NIT1A and NIT1B were kindly provided by Dr. Kazuo Yamaguchi (Institute for Gene Research, Kanazawa University, Kanazawa, Japan), and cloning into pET-21b (+) (Novagen, Madison, WI) was done as described for A. thaliana NIT4 (4). Mutations were introduced using the GeneEditor in vitro Site-directed Mutagenesis System (Promega, Mannheim, Germany).

Expression and Purification of Nitrilase 4 Enzymes—The Escherichia coli strain BL21 (DE3) was used for expression of plant nitrilases. Bacteria grown overnight (600 ml) were collected by centrifugation (5000 × g, 5 min) and resuspended in Buffer A (50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.1% (w/v) β-mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF). After 45 min incubation on ice, the cell suspension was centrifuged (14 000 × g, 20 min) and the supernatant was filtered through a 0.45-μm filter and used as the crude enzyme source.

NHase, nitrile hydratase; PFDOA, pentadecafluoro-octanoic acid; PPN, 3-phenylpropionitrile; DTT, dithiothreitol.
min, 4 °C) and reusupended in 60 ml of lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, 1 mg ml\(^{-1}\) lysosome). Lysis was carried out on ice for 30 min and was completed with six bursts of ultrasound (1 min, 40 watts) using an ultrasound tip (Sonifier B-17, Branson). The 10,000 \(\times g\) supernatant (10 min, 4 °C), containing soluble nitrilase protein, was used for \((NH_4)_2SO_4\) precipitation (40% saturation), and the precipitate was reusupended in 12 ml of lysis buffer omitting lysosome. This fraction was used for purification of the hexahistidine-tagged nitrilases using a 0.5-ml column of Ni\(^{2+}\)-nitrilotriacetic acid-agaroase (Qiagen, Hilden, Germany). Nitrilase bound to the column was eluted with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole. One ml of the peak fraction was collected and desalted using a NAP-10 column (Amersham Pharmacia Biotech) which was equilibrated in 50 mM potassium phosphate, pH 8.0, 1 ml DTT. The resulting nitrilase fraction was purified at least to 95% homogeneity as indicated by Coomassie Blue-stained SDS gels. Protein concentration varied between 80 and 120 \(\mu\)g ml\(^{-1}\), and total volume was 1.5 ml. The protein was shock-frozen in liquid nitrogen and stored at \(-80^\circ\)C for a maximum duration of 8 weeks.

Preparation of Plant Extracts—One gram of plant material was ground to a fine powder in a mortar with liquid nitrogen and thawed with continuously grinding in 3 ml of 100 mM potassium phosphate buffer, pH 8.0, 1 m DT EDTA, 1 mM DTT. The homogenate was centrifuged (15 min, 10,000 \(\times g\), 4 °C), and the supernatant was again centrifuged (20 min, 100,000 \(\times g\), 4 °C). The resulting supernatant (soluble proteins) was brought to 40% saturation of \((NH_4)_2SO_4\) by adding a 100% saturated \((NH_4)_2SO_4\) solution dropwise. After stirring on ice for 20 min, precipitated proteins were collected by centrifugation (15 min, 10,000 \(\times g\), 4 °C) and resuspended in a small volume (0.5–2 ml, depending on the size of the pellet) of 100 mM potassium phosphate buffer, pH 8.0, 1 mM EDTA, 1 mM DTT. Because extracts of blue lupine seedling have a high Asn content, they were desalted in the same buffer using PD-10 columns (Amersham Pharmacia Biotech) before and after the \((NH_4)_2SO_4\) precipitation.

Colorimetric Determination of Nitrilase and Nitrile Hydratase Activity—Nitrilase activity was determined by analyzing the released ammonia using the Bertholet reaction as described previously (4). In brief, the substrate (3 mM) was incubated with 400–600 ng of purified protein for 10–60 min at 30 °C in total reaction volume of 1 ml. The reaction was stopped by adding 1 ml of a mixture of 0.02 M sodium hypochlorite, and 0.01% (w/v) sodium pentacyanonitrosyl ferrate(III) (sodium nitroprusside). After heating for 2 min at 95 °C, the resulting nitrilase fraction was analyzed by liquid chromatography coupled to electrospray-ionization mass spectrometry (LC-ESI-MS). After the indicated times, 0.1 ml aliquots were withdrawn from the reaction vessels, and 100% (v/v) ethanol was added. The samples were boiled for 10 min and subsequently centrifuged (15 min at 13,000 rpm in a tabletop centrifuge) to collect insoluble material. The supernatant was evaporated to dryness and subsequently reusupended in 0.5 ml of 0.5 mM pentadecafluorooctanoic acid (PDFOA). After a second centrifugation, the supernatant was transferred to a fresh reaction tube. Reverse phase liquid chromatography of the undervatized amino acids was carried out according to Chaimbault et al. (14) using a Luna C18(2) column (0.5 \(\mu\)m, 1 × 150 mm, Phenomenex, Aschaffenburg, Germany) on a Waters Cap-LC (Waters, Milford, MA). The injection volume was 5 \(\mu\)l, and the chromatography proceeded isocratically in 0.5 mM PDFOA at a flow rate of 40 \(\mu\)l min\(^{-1}\). The identification of eluting Ala(CN), aspartic acid, and asparagine was achieved by ESI-MS on a Q-TOP2 (Micromass, Manchester, UK) operated in positive ion mode with the following settings: capillary, 3000 V; cone voltage, 16 V; collision energy, 5 eV; collision gas off; MS profile, 113, 113, 113. Expected \(m/z\) values were 115 [Ala(CN) + H\(^+\)], 133 [Asn + H\(^+\)], and 134 [Asp + H\(^+\)]. Quantification was done using external standards and was assisted by the Masslynx software (version 3.4, Micromass, Manchester, UK). To obtain collision-induced decomposition spectra of the amino acids, the automatic MS/MS switching option of the Masslynx software was used with collision gas on and collision energy raised to 15 eV in the MS/MS mode. Signals for the spectra were recorded from \(m/z\) values of 30–140.

RESULTS

Nitrilase 4 Isoforms Are Not Restricted to Brassicaceae—Partial or complete cDNA or genomic sequences of nitrilases are known from several plant species like A. thaliana (1–3), Brassica campestris (Chinese cabbage) (15, 16), Lotus japonicus (GenBankTM accession number AW270658), N. tabacum (tobacco) (8), and Orzya sativa (rice) (GenBankTM accession number AB027054). By comparing the homologies between these nitrilases (Fig. 1), they can be divided into two groups. The first group, referred to as NIT1 group, seemed to be specific for nitrilases from Brassicaceae. Because many species of the Brassicaceae are characterized by their high glucosinolate content and glucosinolate-derived nitriles are among the best substrates for NIT1-NIT3, a function of these enzymes in glucosinolate metabolism has been proposed (4). Arabidopsis NIT4 belongs to the second group, further referred to as NIT4 group to which, in addition to the NIT4s of the Brassicaceae, all known nitrilases of other plants belong. This clustering of the NIT4 sequences could also be observed by phylogenetic analysis using the PHYLIP software package (Dr. J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA) (data not shown). The NIT4 homologs therefore have to be considered as orthologs which means that the present NIT4 genes share a common ancestor. It therefore seems likely that members of the NIT4 group may also have a conserved function.
Plant NIT4 Encodes β-Cyano-α-alanine Hydratase/Nitrilase

NIT4 was purified as His tag fusion protein from E. coli. Protein amounts from 400 to 2400 ng were incubated with the substrates (3 mM) in a volume of 1 ml at 30 °C for 20 min to 4 h. Nitrilase activity was determined colorimetrically as described under “Experimental Procedures.” A specific activity of 100% corresponds to 530 nkat (mg protein)-1. Activity not detectable (in alphabetical order): 2-aminoacetanilide, benzamidine, 2-chloroacetamide, cyanacetamide, α-cyanoaminic acid, 1-cyano-1-cyclopropane carboxylic acid, 6-cyanoaniline, 2-cyanoanidine, 3-cyanoquinidine, 4-cyanoquinidine, cyclopropenecarbonitrile, glutamine, 4-hydroxybenzonitrile, 3-hydroxypropionitrile, indole-3-acetonitrile, indole-3-carbonitrile, mandelonitrile, naphthalene-1-carbonitrile, (phenylthio)acetonitrile, and propionitrile.

| Substrate                  | Relative activity % |
|----------------------------|---------------------|
| β-Cyano-α-alanine          | 100                 |
| L-Asparagine               | 1.5 ± 0.5           |
| 3-Phenylpropionitrile      | 0.75                |
| Phenyacetanilide           | 0.23                |
| (Methylthio)acetanilide    | 0.21                |
| Chloroacetanilide          | 0.07                |
| 3-Aminopropionitrile       | 0.06                |
| Allylycyanide              | 0.03                |
| 4-Phenylbutyronitrile      | 0.02                |

*For details see text.

bacterially expressed protein was found in the 10,000 g sediment, a fraction of the recombinant nitrilase could be purified from the soluble protein lysate by metal-chelate affinity chromatography on nickel-nitrilotriacetate columns. The resulting fraction was purified at least to 95% homogeneity as judged by Coomassie Blue-stained SDS gels. We tested more than 25 selected substrates using the purified enzyme because in preliminary experiments we observed striking and qualitatively different results when the purified enzyme or a crude extract of NIT4-expressing E. coli was used. The results show that NIT4 is highly specific for β-cyano-α-alanine (Ala(CN)) (Table I). The activity of NIT4 against 3-phenylpropionitrile (PPN) or allylycyanide, which are the best substrates for NIT1-NIT3 (4), was very low, and indole-3-acetonitrile (IAN), a precursor of the plant hormone indole-3-acetic acid (IAA), was not detectably converted by NIT4. This is in agreement with in planta data of Schmidt et al. (7) and Dohmoto et al. (17) who were unable to elicit an auxin response with IAN in wild type tobacco (which expresses at least two NIT2 homologs) as well as in NIT4-overexpressing tobacco (17). Transgenic tobacco plants expressing either NIT2 (7), NIT1 (17), or NIT3 (17) of A. thaliana converted this substrate to IAA and developed strong phenotypic symptoms of auxin overproduction.

NIT4 Is Both a Nitrilase and a Nitrile Hydratase—Since the enzymatic assay used so far was based on the analysis of released ammonia, it was necessary to show that this ammonia represented the nitrile nitrogen and not the amino nitrogen. Thus, we analyzed the reaction products by thin layer chromatography (data not shown) and liquid chromatography coupled to mass spectrometry (LC-ESI-MS) (Fig. 2). The production of aspartic acid (Asp) could unequivocally be shown by co-chromatography with authentic Asp during TLC (data not shown) as well as by LC-ESI-MS (Fig. 2A) and by its collision-induced decomposition spectrum (Fig. 2B). Unexpectedly, Asn could also be detected and occurred in amounts about 1.5 times higher than Asp. Therefore, NIT4 not only has a nitrilase activity (converting Ala(CN) to Asp) but also a nitrile hydratase (NHase) activity (converting Ala(CN) to Asn). Nitrilases hydrolyze nitriles by the successive addition of two molecules of water, whereas the substrate remains covalently bound to the enzyme’s catalytic-site cysteine (18). Our results suggested that Asn may occur as a free intermediate of the nitrilase reaction rather than enzyme-bound. However, this could be ruled out by the observation that Asn is only marginally converted to Asp by NIT4 (Table I). This negligible asparaginase activity of NIT4 (6 nkat (mg protein)-1) cannot account for the observed levels of Asp that is formed at a rate of ~500 nkat (mg protein)-1. Furthermore, asparaginase activity was not observed in every preparation and results therefore most likely from small contaminations of the preparations.

**TABLE I**

| Substrate                  | Relative activity % |
|----------------------------|---------------------|
| α-Cyano-L-alanine          | 1.5 ± 0.5           |
| 3-Phenylpropionitrile      | 0.75                |
| Phenyacetanilide           | 0.23                |
| (Methylthio)acetanilide    | 0.21                |
| Chloroacetanilide          | 0.07                |
| 3-Aminopropionitrile       | 0.06                |
| Allylycyanide              | 0.03                |
| 4-Phenylbutyronitrile      | 0.02                |

2 R.-C. Schmidt, unpublished data.
with *E. coli* asparaginase. In addition, time course studies showed that Asp and Asn are formed at the same time with a constant ratio with no detectable turnover of Asn (Fig. 3). If Asn would indeed be an intermediate of the reaction, we would expect it to accumulate before Asp synthesis starts, whereas with the onset of Asp formation its level should decrease. Taken together, these results prove that NIT4 converts Ala(CN) to either Asp or Asn. Asn is no substrate of the enzyme, and thus, Asn is no free intermediate of the nitrilase reaction of this bifunctional enzyme.

**Is the Catalytic Site for Nitrilase and NHase Activity the Same?**—The known reaction mechanisms of nitrilases and NHases are quite different (for review see Ref. 19). Although the first bind their substrate covalently to a cysteine residue, the latter use a nonheme iron for their activity. The formation of Asn from Ala(CN) by NIT4 may be the result of a "premature" release of Asn during the nitrilase reaction or it may occur at a second active site with NHase activity. The substrate concentration dependence of the two reactions (Fig. 4 and Table II) revealed that the *K*<sub>m</sub> of Ala(CN) for both reactions is very similar if not identical, whereas the maximum velocity (*V*<sub>max</sub>) is higher for the Asn formation. This result suggests that the catalytic center for both reactions might be the same. Both reactions showed the same temperature and pH dependence (Table II), and they were both inhibited by N-ethylmaleimide (100% inhibition at 2 mM). The involvement of a cysteine residue in both reactions was further indicated by their inhibition at higher concentrations of DTT (~50% inhibition at 10 mM). To pinpoint this residue(s), the proposed catalytically active cysteine of NIT4 for the nitrilase reaction (Cys-197) was mutated to alanine. The mutant protein (NIT4C197A) showed no nitrilase activity, and a strongly inhibited NHase activity (~5% of the wild type protein) (Fig. 2) demonstrating (i) the importance of Cys-197 for both activities but also (ii) that the NHase activity does not completely depend on this residue.

**Is the NHase Activity of NIT4 Genuine?**—In 1995, Dufour *et al.* (20) showed that a single amino acid substitution (Gln to Glu) in the cysteine protease papain resulted in a novel NHase activity of the mutated protein. The observed NHase activity of *A. thaliana* NIT4 could therefore be the result of an artificial mutation. Sequence errors could be ruled out because the reported genomic and cDNA sequences of NIT4 from *A. thaliana* encode the same polypeptide. Additionally, NHase activity was seen with three different NIT4 homologs (see below). A critical factor may be the intentional "mutation" of the C terminus of the enzyme introduced with the His tag (EVHHHHHHH) that was used in all tested recombinant NIT4 proteins. To study the influence of the His tag, we expressed NIT4 using its genuine stop codon in *E. coli* and enriched the protein by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation and gel filtration. NIT4 activity was found in the void volume of a Superdex 200 HiLoad 16/60 column (Amersham Pharmacia Biotech) indicating that the native molecular mass is greater than 600 kDa. The active fractions showed no asparaginase activity under the conditions applied, but both nitrilase and NHase activities could be detected, displaying a ratio of 1:1.25 like the His-tagged protein (data not shown). The NHase activity is therefore an intrinsic property of the NIT4 protein.

**β-Cyanoalanine Hydrolysis Is a Common Feature of NIT4 Proteins**—As mentioned above, proteins homologous to NIT4 are also known from tobacco (8) and rice. The cDNAs of the tobacco nitrilases TNIT4A and TNIT4B were kindly provided to us by Dr. Kazuo Yamaguchi (Institute for Gene Research,
Comparison of Arabidopsis NIT4 with tobacco TNIT4A and TNIT4B

NIT4, TNIT4A, and TNIT4B were purified as His tag fusion proteins from *E. coli*. Protein amounts of 400–2400 ng were incubated with the substrate (3 mM) in a volume of 1 ml at 37 °C for 20 min to 2 h. Activity was determined colorimetrically or by LC-ESI-MS as described under “Experimental Procedures.” Results are means ± S.D. of three independent experiments.

|        | NIT4   | TNIT4A | TNIT4B |
|--------|--------|--------|--------|
| ratio NHase/nitrilase activity | 1.36 ± 0.21 | 0.87 ± 0.04 | 1.06 ± 0.12 |
| ratio Ala(CN)/PPN nitrilase activity | 119 ± 18 | 28 ± 8 | 20 ± 4 |

Kanazawa University, Kanazawa, Japan. By using the same cloning strategy as used for *A. thaliana* NIT4, both cDNAs could be expressed in *E. coli*, and the proteins were purified using Ni

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**DISCUSSION**

The occurrence of nitrilases in higher plants is known since 1972, when Cas- 

tric et al. (10) reported the characterization of a β-cyanoalanine hydratase enriched from blue lupine seedlings (*L. angustifolius*), which formed Asn from Ala(CN). It is not entirely clear from this paper if the authors also looked for Asp production (Table III) compared with the Arabidopsis enzyme, thus producing relatively more Asp. Interestingly, PPN was a better substrate for the tobacco enzymes than for Arabidopsis NIT4, the ratio of AlacN to PPN consumption was ~5–6 times higher with the tobacco enzymes (Table III). As already mentioned, *A. thaliana* has a high content of glucosinolates, and glucosinolate-derived nitriles are substrates for the Arabidop-

sis isoenzymes NIT1, NIT2, and NIT3. It is therefore possible that Arabidopsis NIT4 lost PPN hydrolase activity during evolu-

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**Fig. 5. NIT4 activity in extracts of different plants.** Protein was extracted from 1 g of tissue (*L. angustifolius*, 5-day-old light-grown seedlings; *N. tabacum*, the oldest leaf of a 3-month-old plant; *A. thali-

na*, nonsenescent (ns) rosette leaves of nonbolting plants or senescent (s) rosette leaves of flowering plants) and enzymatic activity enriched by (NH

4)

2SO4 precipitation (40% saturation); 50–100 µg of protein were incubated with 3 mM Ala(CN) at 37 °C for 3 h. Nitrilase and NHase activity were determined by LC-ESI-MS as described under “Experi-

mental Procedures.” The data shown are means ± S.D. from three experiments.

**Table III**

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**Plant NIT4 Encodes β-Cyano-L-alanine Hydratase/Nitrilase**

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**ability to convert indole-3-acetonitrile to the plant hormone indole-3-acetic acid (1, 3, 22).** Genomic or cDNA sequences of nitrilases are now known from *A. thaliana*, two Brassica species (*B. campestris* and *B. oleracea*) (15, 16), tobacco (*N. tabacum*) (17), and rice (*O. sativa*, GenBank access-

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**Fig. 5.** NIT4 activity in extracts of different plants. Protein was extracted from 1 g of tissue (*L. angustifolius*, 5-day-old light-grown seedlings; *N. tabacum*, the oldest leaf of a 3-month-old plant; *A. thali-

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mental Procedures.” The data shown are means ± S.D. from three experiments.

**Table III**

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**Comparison of Arabidopsis NIT4 with tobacco TNIT4A and TNIT4B**

NIT4, TNIT4A, and TNIT4B were purified as His tag fusion proteins from *E. coli*. Protein amounts of 400–2400 ng were incubated with the substrates (3 mM) in a volume of 1 ml at 37 °C for 20 min to 2 h. Activity was determined colorimetrically or by LC-ESI-MS as described under “Experimental Procedures.” Results are means ± S.D. of three inde-

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**DISCUSSION**

The occurrence of nitrilases in higher plants is known since 1958 (22), but until now the main interest was directed to their
Plant NIT4 Encodes β-Cyano-L-alanine Hydratase/Nitrilase

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ing from the known mechanism of "classical" NHases. Elucidating this mechanism will be of value for the design of new nitrile-degrading enzymes that could be of use for environmental and industrial applications. A possible explanation for this NHase activity may be a "premature" release of the enzyme-bound substrate after the addition of the first molecule of water. If the second molecule of water is not delivered fast enough, the amide may be released, whereas, if the water is present, the substrate will be processed further to the acid.

Interestingly, an enzyme showing the same enzymatic characteristics as NIT4 was purified from Pseudomonas sp. 13 in 1983 (23), but no sequence of this protein was reported until now. The reported biochemical data of this enzyme are very similar to NIT4, but its $K_m$ for Ala(CN) is about 1 order of magnitude higher compared with Arabidopsis NIT4.

Ala(CN) is a product of cyanide detoxification of plants. It is produced from cyanide and cysteine by cyanolalanine synthase (9). Recent results indicate that cyanolalanine synthase is an enzyme homologous or identical to mitochondrial cysteine synthase (24, 25). In most species analyzed, Ala(CN) is then converted to Asn, although in some species it is converted to the dipeptide γ-glutamyl-cyanoalanine (γ-Glu-Ala(CN)). The enzyme(s) catalyzing the formation of Asn from Ala(CN) (cyanoalanine hydratase = Ala(CN) NHase) were biochemically studied from lupine seedlings in the laboratory of E. Conn in the early 70s (10) and later by Galoyan et al. (11), but until now, genes encoding such enzymes have not been cloned, and characterizations of corresponding enzymes from other plant species are lacking. Is Conn's cyanoalanine hydratase and NIT4 the same enzyme? In several feeding experiments using $^{14}$CN, radioactive label could be detected in Asn but Asp was not significantly labeled (10, 26) arguing against a NIT4-catalyzed reaction in which similar amounts of Asp and Asn would be expected. We therefore tested the ability of plant extracts from A. thaliana, tobacco, tomato, L. sativa, B. dioica, and blue lupine to hydrolyze Ala(CN) and could unequivocally detect Ala(CN) NHase activity as well as Ala(CN) nitrilase activity in all tested extracts. In extracts of blue lupine the Ala(CN) NHase activity was dominant; nevertheless, nitrilase activity was clearly detectable. It is therefore likely that the Ala(CN) NHase from blue lupine is a NIT4 homolog. This topic is currently under investigation in our laboratory. Our in vitro data are, however, in contrast to the data obtained in vivo from the $^{14}$CN labeling experiments mentioned above. A possible explanation could be that the turnover rate of Asp is higher than that of Aan in vivo, as observed in cotton roots (26). In this case Asp would not accumulate.

One possible source of cyanide in higher plants is the biosynthesis of the plant hormone ethylene from 1-aminocyclopropane-1-carboxylic acid (27). During this reaction cyanoformic acid is produced which then spontaneously degrades to carbon dioxide and cyanide. Interestingly, the Arabidopsis NIT4 promoter was found to be activated during leaf senescence as shown by Northern blot analysis (20). We detected severalfold higher NIT4 activity in extracts from senescent leaves of A. thaliana compared with extracts from nonsenescent leaves, and the ratio of NHase to nitrilase activity decreased (Fig. 5). This change may indicate that different enzymes are involved in Ala(CN) metabolism at different developmental stages, but it may also indicate that NHase and nitrilase activity of NIT4 could be regulated independently, e.g., by posttranslational modifications. Whether NIT4 activity or NIT4 expression is connected to ethylene biosynthesis or cyanide production during leaf senescence will be addressed in future studies.

The results presented in this paper clearly show that NIT4 enzymes from A. thaliana and N. tabacum are Ala(CN) hydrotases/nitrilases. NIT4 homologs are known from several different species of quite different taxonomical position, and it is likely that NIT4 homologs may be present in all higher plants. NIT4 is proposed to take part in cyanide detoxification in vivo in cooperation with Ala(CN) synthase. We propose to reserve the gene name NIT4 for Ala(CN) hydrotases/nitrilases. Nitrilases from other plants belonging to the NIT4 family should therefore also be called NIT4 independent of the total number of NIT genes present, as previously done for B. campestris (15), tobacco (8), and rice.

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