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Molecular characterization of two cloned nitrilases from *Arabidopsis thaliana*: Key enzymes in biosynthesis of the plant hormone indole-3-acetic acid

(auxin/differential expression/nucleotide sequence/indoleacetonitrile/plasma membrane)

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ABSTRACT **As in maize [Wright, A. D., Sampson, M. B., Neuffer, M. G., Michalczuk, L., Slovin, J. P. & Cohen, J. D. (1991) *Science* 254, 998–1000], the major auxin of higher plants, indole-3-acetic acid, is synthesized mainly via a nontryptophan pathway in *Arabidopsis thaliana* [Normanly, J., Cohen, J. D. & Fink, G. R. (1993) *Proc. Natl. Acad. Sci. USA* 90, 10355–10359]. In the latter species, the hormone may be accessible from the glucosinolate glucobrassicin (indole-3-methyl glucosinolate) and from L-tryptophan via indoleacetaldoxime under special circumstances. In each case, indole-3-acetonitrile is the immediate precursor, which is converted into indole-3-acetic acid through the action of nitrilase (nitrile aminohydrolase, EC 3.5.5.1). The genome of *A. thaliana* contains two nitrilase genes. Nitrilase I had been cloned earlier in our laboratory. The cDNA for nitrilase II (PM255) was cloned and encodes an enzyme that converts indole-3-acetonitrile to indole-3-acetic acid, the plant hormone. We show that the intracellular location as well as the expression pattern of the two *A. thaliana* nitrilases are distinctly different. Nitrilase I is soluble and is expressed throughout development, but at a very low level during the fruiting stage, while nitrilase II is tightly associated with the plasma membrane, is barely detectable in young rosettes, but is strongly expressed during bolting, flowering, and especially fruit development. The results indicate that more than one pathway of indole-3-acetic acid biosynthesis via indole-3-acetonitrile exists in *A. thaliana* and that these pathways are differentially regulated throughout plant development.**

While the structure of the phytohormone indole-3-acetic acid (IAA), the predominant auxin that regulates numerous aspects of plant life (1), was elucidated 60 years ago, IAA biosynthesis and its regulation are still incompletely understood. Several pathways of IAA biosynthesis have been reported for different plants (2) or even at different stages of development (3), but rigorous establishment of the process has not been accomplished. It is now clear that tryptophan is not always a precursor of IAA (4–6), but the details of this unusual pathway, established for the *orp* mutant of maize (5) and, recently, for *Arabidopsis thaliana* (6), are not yet known. *A. thaliana* lends itself particularly to the study of IAA biosynthesis because of the availability of several mutants in the tryptophan biosynthetic pathway (6–8). From precursor feeding experiments using the *A. thaliana trp2-1* (tryptophan synthase β deficient) and *trp3-1* (tryptophan synthase α deficient) mutants, Normanly *et al.* (6) showed recently that anthranilate, but not L-tryptophan, was a major precursor to IAA. The pool of endogenous indole-3-acetonitrile (IAN) as well as that of IAA was increased in these mutants, and IAN carried label from anthranilate, as expected for the IAA precursor (6). Minor contributions to the pool of IAA from tryptophan via the indoleacetaldoxime pathway proposed earlier (9) could not be excluded completely in this study (6). IAN is also a proposed intermediate in this pathway. A third route to IAA may lead from myrosinase-catalyzed degradation of indole-3-methyl glucosinolate (glucobrassicin) via IAN to the auxin, but this pathway may occur only at specific stages in plant development (10). Glucobrassicin is a major glucosinolate in *A. thaliana*, especially in the seeds (11).

These data suggest multiple pathways to IAA in *A. thaliana*, all involving IAN as the direct auxin precursor. Nitrilase (nitrile aminohydrolase, EC 3.5.5.1) must thus be regarded as the key enzyme in the biosynthesis of IAA in this species. Nitrilase I has been cloned in our laboratory (12), but Southern hybridizations showed the presence of a second nitrilase gene in this plant. We have now cloned and functionally expressed a cDNA encoding this second enzyme, and we show that the two nitrilases, while similar in their enzymatic properties, are localized in different intracellular compartments and that their expression is differentially regulated during plant development.† The results are consistent with the occurrence of at least two differently regulated and expressed IAN-dependent pathways of IAA biosynthesis in a higher plant.

MATERIALS AND METHODS

Plant Material. *A. thaliana* (L.) Heynh. (ecotype Landsberg erecta) was grown as described (12). Aseptic plants, cultivated at 22°C, 50 $W \cdot m^{-2}$, and 8-h photoperiod on 50% Murashige–Skoog medium (13) containing 0.5% (wt/vol) Gelrite (Kelco, Schweizerhall, South Plainfield, NJ), but no hormones, were used throughout.

Preparation of Subcellular Fractions and Marker Enzyme Assays. Microsomes and purified plasma membrane vesicles were prepared from whole plants as described (14, 15). Freshly prepared plasma membrane vesicles were centrifuged for 3 h at 4°C and 90,000 × *g* on sucrose density gradients, and the fractions were assayed for marker enzymes (16) as well as subjected to $NaDodSO_4$/PAGE followed by immunoblot analysis with anti-nitrilase antibody (see below).

Expression of Nitrilases in *Escherichia coli*. Enzymically active nitrilases from the clones PM81 and PM255 were obtained in the *E. coli* strain XL-1 Blue (Stratagene) (12). For protein overexpression, the *Eco*RI/*Kpn* I restriction fragment containing the full-length cDNA PM81 was cloned into

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Abbreviations: IAN, indole-3-acetonitrile; IAA, indole-3-acetic acid.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X68305).

the expression vector pEXP2 (17) and transformed into *E. coli* strain W3110 (18). Protein was extracted from inclusion bodies and the nitrilase protein was purified by $NaDodSO_4$/PAGE and electroelution (19).

Assays for Nitrilase Activity. The enzymatic activity of nitrilase was determined in bacterial extracts with IAN as substrate (12). Verification of the structure of the reaction product was done as follows. Acidic ethyl acetate fractions from enzymatic reactions were obtained and purified by high-performance liquid chromatography (12). Fractions corresponding to the retention time of IAA (12.9 min) were subjected to capillary gas chromatography/mass spectroscopy using a Finnigan Magnum ion trap mass spectrometer run in electron impact (70 eV) mode. Indoles were separated on a WCOT fused silica column (DB-17; J & W Scientific, Rancho Cordova, CA) (30 m × 0.25 mm i.d.) (0.25-μm coat). Enzyme activity in plant samples was determined by quantitation of the IAA formed in a monoclonal antibody-based enzyme-linked immunosorbent assay (12, 20).

Immunological Methods. Antibodies were affinity purified from antisera raised against the purified nitrilase encoded by cDNA PM81 expressed from pEXP2 (21).

Cloning of cDNA and Molecular Characterization of Nitrilase PM255. The cDNA PM255 was identified by its hybridization to full-length PM81 (12) probes. Plasmids were sequenced from the 5′ end using the dideoxynucleotide chain-termination procedure (22) following the Sequenase (Stratagene) protocol and aligned by the DNASIS program (Pharmacia). The PM255 cDNA was completely sequenced on both strands from multiple-overlapping partial clones generated by restriction enzymes *Taq* I and *Msp* I and subcloning of fragments. Amino acid sequences were aligned by using the PROSIS (Pharmacia), FASTP (EMBL), and CLUSTAL V (23) programs.

RNA Blot Analysis. Total RNA was extracted from whole, aseptically grown plants representing different developmental stages, and poly(A)$^+$ RNA was isolated by standard procedures (19). Poly(A)$^+$ RNA was separated on 1.2% agarose gels and blotted onto GeneScreen nitrocellulose membranes (DuPont) using Turbo-blot chambers (Schleicher & Schuell) and 10× SSPE (19) as transfer buffer. RNA blots were prepared according to the Southern-Light, version J, protocol (Tropix) using 0.02 μg of Luminol per ml (Boehringer) as substrate.

RESULTS

***A. thaliana* Nitrilases Occur in Different Cellular Locations.** The nitrilase antiserum, affinity purified against the overexpressed nitrilase on Western blots (21), detected a single polypeptide with the expected molecular mass (39.8 kDa) in crude protein extracts of induced bacteria and labeled a single band of 35.5 kDa in the total protein fraction from *A. thaliana* plants, in agreement (within methodological limits) with the molecular mass (37.5 kDa) of nitrilase calculated from the primary sequence (Fig. 1, lanes 1 and 2). The nitrilase polypeptide was associated with microsomal membranes and, within this fraction, specifically with the plasma membrane (lanes 4 and 5). It was absent from chloroplasts (total protein loaded; lane 6) but a signal was also detected in the fraction of soluble proteins (lane 3). During aqueous two-phase partitioning of microsomal membranes, nitrilase activity and polypeptide copurified with the plasma membrane marker H^+-ATPase (Table 1). The plasma membrane-bound enzyme was tightly associated with this structure, as it could not be removed by high salt and repeated washings (data not shown). When partitioned against Triton X-114 (25), the polypeptide accumulated in the aqueous phase. The plasma membrane-associated nitrilase, by this criterion, classified as a peripheral membrane protein. Further fractionation experiments using

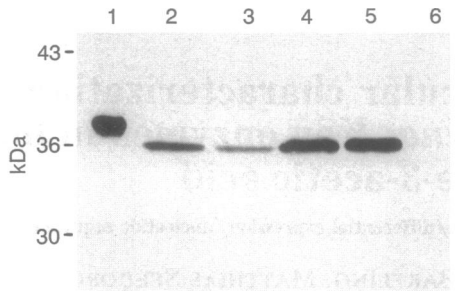

FIG. 1. Subcellular distribution of nitrilase polypeptide, determined by immunodetection after separation of proteins on $NaDodSO_4$/polyacrylamide gels. Per lane, 60 μg of protein was loaded. Lanes: 1, bacterially expressed nitrilase encoded by cDNA PM81 (whole cell protein); 2–6, protein from *A. thaliana* plants; 2, total protein; 3, total soluble protein; 4, microsomal protein; 5, purified plasma membrane protein; 6, total chloroplast protein. Chloroplasts were prepared according to ref. 24.

discontinuous sucrose density gradients proved that in addition to the plasma membrane-bound enzyme, a substantial fraction of nitrilase activity and polypeptide were soluble (data not shown). The relative amounts of soluble versus membrane-bound nitrilase were dependent on plant age and stage of differentiation (see below). Thus, the biochemical data indicated the presence of two nitrilases localized in different cellular compartments. Southern blots had earlier also suggested the presence of two homologous nitrilase genes in the *A. thaliana* genome (12).

Cloning and Sequencing of cDNA Encoding Nitrilase II of *A. thaliana*. Clone PM255 was identified because it strongly hybridized to PM81 cDNA, yet it exhibited different restriction fragment patterns. The sequence of PM255 is 1305 bp long and contains an open reading frame (encompassing nucleotides 21–1037) that would encode a protein of 339 amino acids with a calculated molecular weight of 37,151 (Fig. 2).

In addition to the enzymes from *A. thaliana*, only two other nitrilases have been cloned, both of them from prokaryotes (26, 27). The deduced amino acid sequences of the plant enzymes and those from *Klebsiella ozaenae* (encoded by the *bxn* gene) (26) and *Alcaligenes faecalis* JN3 (27) are aligned in Fig. 3. The two plant enzymes share 90.3% identical amino acids, while PM255 is only 33.7% identical to the *Klebsiella* sequence and 27.4% identical to that of *Alcaligenes*. The sequences of the two enzymes from prokaryotes show 33.7% identity. The consensus sequence for all four nitrilases is also shown in Fig. 3. It can be seen that contiguous stretches of identity do not exist; however, conserved amino acid residues are spaced all over the proteins. The active site cysteine (Cys-163 in JN3) (27) is conserved in all four sequences, and it is the only conserved cysteine (Cys-179 in the PM255 and

Table 1. Microsomal nitrilase is a plasma membrane enzyme as shown by phase partitioning (14)

Parameter	Relative distribution, %			
	L1	L2	L3	U
Protein	71.3	7.1	2.4	19.2
Chlorophyll	91.6	6.2	0.0	2.2
NADH cytochrome *c* reductase	56.0	9.2	5.1	29.7
Succinate dehydrogenase	75.5	2.8	8.9	12.8
Proton ATPase	27.5	9.7	4.1	58.6
Nitrilase activity	23.3	12.6	6.0	58.1
35.5-kDa polypeptide detected by nitrilase antiserum				

L1–L3, lower phases; U, final upper phase containing purified plasma membranes.

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CTCAAGTTAAAACAGAAAAAATGTCAACTTCAGAAAACACTCCGTTTAATGGCGTTGCC    59
                  M  S  T  S  E  N  T  P  F  N  G  V  A         13

TCATCCACCATTGTTCGAGCTACCATTGTCCAAGCCTCCACCGTCTACAACGATACTCCC   119
 S  S  T  I  V  R  A  T  I  V  Q  A  S  T  V  Y  N  D  T  P    33

GCCACTCTAGAAAAGGCGAACAAGTTTATTGTGGAGGCTGCAAGCAAGGGATCGGAGCTG   179
 A  T  L  E  K  A  N  K  F  I  V  E  A  A  S  K  G  S  E  L    53

GTTGTGTTCCCGGAGGCGTTTATCGGTGGTTATCCTCGAGGTTTTAGGTTTGGTTTAGGG   239
 V  V  F  P  E  A  F  I  G  G  Y  P  R  G  F  R  F  G  L  G    73

GTCGGAGTTCATAACGAAGAAGGGCGTGATGAGTTCCGCAAGTACCATGCTTCTGCTATT   299
 V  G  V  H  N  E  E  G  R  D  E  F  R  K  Y  H  A  S  A  I    93

AAAGTTCCTGGCCCTGAAGTAGAAAAGTTGGCGGAGTTGGCCGGGAAGAACAATGTGTAC   359
 K  V  P  G  P  E  V  E  K  L  A  E  L  A  G  K  N  N  V  Y   113

TTGGTAATGGGAGCGATAGAGAAGGATGGGTATACACTCTATTGCACAGCACTTTTCTTC   419
 L  V  M  G  A  I  E  K  D  G  Y  T  L  Y  C  T  A  L  F  F   133

AGTCCACAAGGTCAGTTCTTGGGTAAGCACCGTAAACTCATGCCCACAAGTTTGGAACGT   479
 S  P  Q  G  Q  F  L  G  K  H  R  K  L  M  P  T  S  L  E  R   153

TGCATTTGGGGTCAAGGAGACGGATCAACCATCCCCGTTTACGACACTCCGATTGGAAAA   539
 C  I  W  G  Q  G  D  G  S  T  I  P  V  Y  D  T  P  I  G  K   173

CTCGGTGCTGCTATTTGCTGGGAGAATAGGATGCCCCTCTACAGAACCGCATTGTACGCC   599
 L  G  A  A  I  C  W  E  N  R  M  P  L  Y  R  T  A  L  Y  A   193

AAAGGCATTGAGCTTTATTGTGCACCTACTGCTGATGGTTCGAAAGAATGGCAATCGTCG   659
 K  G  I  E  L  Y  C  A  P  T  A  D  G  S  K  E  W  Q  S  S   213

ATGCTTCACATTGCGATCGAAGGTGGATGTTTCGTATTGTCGGCTTGCCAGTTCTGCCTT   719
 M  L  H  I  A  I  E  G  G  C  F  V  L  S  A  C  Q  F  C  L   233

CGTAAAGATTTCCCTGATCATCCTGACTACTTGTTTACCGATTGGTACGACGACAAAGAG   779
 R  K  D  F  P  D  H  P  D  Y  L  F  T  D  W  Y  D  D  K  E   253

CCTGACTCTATTGTTTCCCAAGGTGGAAGTGTTATTATTTCACCTTTGGGACAGGTTCTT   839
 P  D  S  I  V  S  Q  G  G  S  V  I  I  S  P  L  G  Q  V  L   273

GCGGGACCAAACTTTGAATCAGAGGGTCTCATCACAGCTGATCTTGATCTTGGTGATGTA   899
 A  G  P  N  F  E  S  E  G  L  I  T  A  D  L  D  L  G  D  V   293

GCAAGAGCTAAGTTGTACTTCGATTCGGTTGGACATTACTCGAGACCAGATGTTTTACAC   959
 A  R  A  K  L  Y  F  D  S  V  G  H  Y  S  R  P  D  V  L  H   313

TTGACCGTAAATGAGCACCCGAAGAAACCGGTCACATTCATTTCGAAGGTGGAGAAAGCG  1019
 L  T  V  N  E  H  P  K  K  P  V  T  F  I  S  K  V  E  K  A   333

GAAGATGACTCAAACAAGTAATCGGTTGTGATTCGTCAGTTCATGTCACTCCTATGAAGG  1079
 E  D  D  S  N  K  *

AGTCAAGTTCAAAATGTTATGTTGAGTTTCAAACTTTTATGCTAAACTTTTTTTCTTTAT  1139
TTTCGTTAATAATGGAAGAGAACCAATTCTCTTGTATCTAAAGATTATCCATCTATCATC  1199
CAATTTGAGTGTTCAATTCTGGATGTTGTGTTACCCTACATTCTACAACCATGTAGCCAA  1259
TTATTATGAATCTGGCTTTGATTT(A)22
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FIG. 2. Nucleotide and deduced amino acid sequence of the cDNA PM255. The first base of the cDNA is designated as position 1. Arrow indicates beginning of a 304-bp *Xho* I-generated subfragment of PM255 used for synthesis of biotinylated probes for Northern blot analysis (see Fig. 5). Underlined bases indicate positions of possible polyadenylylation signals.

Cys-180 in the PM81 sequence). The region around the catalytic site cysteine is highly conserved in the sequences of PM255 and PM81, while sequence divergence increases toward both the C-terminal and especially the N-terminal regions. It is obvious that PM255 encodes a nitrilase different from the enzyme encoded by cDNA PM81 but is clearly more related to this than to the two bacterial nitrilases, which among themselves are also relatively divergent. For clarity, the enzyme encoded by PM255 is termed nitrilase II, while that encoded by PM81 is termed nitrilase I.

Nitrilase II Encoded by cDNA PM255 Is a Functional Enzyme Converting IAN to IAA. Both cDNAs encoding nitrilases I and II were cloned in the protein expression vector pBluescript SK and maintained in the same bacterial host (*E. coli* W3110) from which, by induction with isopropyl β-D-thiogalactopyranoside, the polypeptides are expressed as fusion proteins containing an N-terminal extension of 53 (PM81) or 48 (PM255) amino acids representing sequences 5′ upstream of the eukaryotic translation start codon and the N-terminal part of β-galactosidase as well as the translated sequences from the multiple cloning site. PM81 was earlier shown to be functionally expressed in this system (12), although the long N-terminal fusion peptide to some extent restricted access of the substrate to the active site, resulting in an increased K_m. Using this vector/host system, we were able to show that PM255 encodes a functional nitrilase, which converts IAN to IAA (Fig. 4). The reaction product was

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PM255      MSTSEN-TPF NGVASSTIVR ATIVQASTVY NDTPATLEKA NKFIVEAASK  49
PM81       MSTVQNATPF NGVAPSTTVR VTIVQSSTVY NDTPATIDKA EKYIVEAASK
bxn        MDT------- -------TFK AAAVQAEPVW MDAAATADKT VTLVAKAAAA
JM3        MQTRK----- -------IVR AAAVQAASPN YDLATGVDKT IELARQARDE
consensus  M.T..... ..........  ...VQ..... .D......K. ......A...

PM255      GSELVVFPEA FIGGYPRGFR FGLGVGVHNE EGRDEFR-KY HASAIKVPGP  98
PM81       GAELVLFPEG FIGGYPRGFR FGLAVGVHNE EGRDEFR-KY HASAIHVPGP
bxn        GAQLVAFPEL WIPGYP---- --GFMLTHNQ TETLPFIIKY RKQAIAADGP
JM3        GCDLIVFGET WLPGYP---- --FHVWLGAP AWSLKYSARY YANSLSLDSA
consensus  G..L..F.E. ...GYP.... .......... .........Y ..........

PM255      EVEKLAELAG KNNVYLVMGA IEKDGYTLYC TALFFSPQGQ FLGKHRKLMP 148
PM81       EVARLADVAR KNHVYLVMGA IEKEGYTLYC TVLFFSPQGQ FLGKHRKLMP
bxn        EIEKIRCAAQ EHNIALSFGY SERAGRTLYM SQMLIDADGI TKIRRRKLKP
JM3        EFQRIAQAAR TLGIFIALGY SERSGGSLYL GQCLIDDKGQ MLWSRRKLKP
consensus  E.......A. ........G. .E..G..LY. ........G. .....RKL.P

PM255      TSLERCIWGQ GDGSTIPVYD TPIGKLGAAI CWENRMPLYR TALYAKG---  195
PM81       TSLERCIWGQ GDGSTIPVYD TPIGKLGAAI CWENRMPLYR TALYAKG---
bxn        TRFERELFGE GDGSDLQVAQ TSVGRVGALN CAENLQSLNK FALAAEGEQI
JM3        THVERTVFGE GYARDLIVSD TELGRVGALC CWEHLSPLSK YALYSQHEAI
consensus  T..ER...G. G......V.. T..G..GA.. C.E....L.. .AL.......

PM255      -------IEL YCAPTADGSK EWQSSMLHI- AIEGGCFVLS ACQFCLRKDF  237
PM81       -------IEL YCAPTADGSK EWQSSMLHI- AIEGGCFVLS ACQFCQRKHF
bxn        HISAWP-FTL ---GSPVLVG DSIGAINQVY AAETGTFVLM STQVVGPTGI
JM3        HIAAWPSFSL YSEQAHALSA KVNMAASQIY SVEGQCFTIA ASSVVTQETL
consensus  .........L .......... .......... ..E...F... ..........

PM255      PDHPDYLFTD WYDDKEPDSI VSQGGSVIIS PLGQVLAGP- NFESEGLITA  286
PM81       PDHPDYLFTD WYDDKEHDSI VSQGGSVIIS PLGQVLAGP- NFESEGLVTA
bxn        ---AAFEIED RYNPNQY--- LGGGYARIYG PDMQLKSKSL SPTEEGIVYA
JM3        ---DMLEVGE HNASLLK--- VGGGSSMIFA PDGRTLAPYL PHDAEGLIIA
consensus  .......... .......... ...G...I.. P......... ....EG...A

PM255      DLDLGDVARA KLYFDSVGHY SRPDVLHLTV NEHPKKPVTF SKVEKAEDD  336
PM81       DIDLGDIARA KLYFDSVGHY SRPDVLHLTV NEHPRKSVTFV TKVEKAEDD
bxn        EIDLSMLEAA KYSLDPTGHY SRPDVFSVSI NRQRQPAVSEV IDSNGDEDP
JM3        DLNMEEIAFA KAINDPVGHY SKPEATRLVL DLGHREPMTRV HSKSVIQEE
consensus  .........A K...D..GHY S.P..... .......... .........

PM255      SNK*                                                    339
PM81       SNK*
bxn        RAACEPDEGD REVVISTAIG ---VLPRYCG HS*
JM3        APEPHVQSTA APVAVSQTQD SDTLLVQ--E PS*
consensus  .......... .......... .......... ..
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FIG. 3. Comparison of amino acid sequences of all nitrilases cloned so far. Amino acid residues different between the two plant nitrilases are indicated by open arrowheads. Gaps necessary for optimum alignment are indicated by dashes. Catalytic center cysteine is marked with a solid circle. PM255, this paper; PM81, nitrilase I from *A. thaliana* (12); bxn, *K. ozaenae* (26); JN3, *A. faecalis* (27); consensus, identical residues in all sequences.

unambiguously identified by full-scan mass spectroscopy following separation of the product by high-performance liquid chromatography and capillary gas chromatography. Control incubations of IAN with bacterial strains harboring an unrelated cDNA (*Ath5* encoding an *A. thaliana* ribulose-1,5-bisphosphate carboxylase small subunit) did not produce detectable amounts of IAA (data not shown; but see ref. 12). Further analysis showed nitrilases I and II expressed from Bluescript SK to have similar enzymatic properties in terms of apparent K_m for IAN (5 mM), V_{max} (2–4 pkat per mg of protein), pH 7.5 optimum, and temperature optimum (30°C) as well as substrate and inhibitor dependence (details to be reported elsewhere).

Nitrilases I and II Are Differentially Expressed During Plant Development. Due to the similar primary sequences of the two *A. thaliana* nitrilases, antibodies raised against enzyme I (12) also recognize nitrilase II. Differential nucleotide probes were derived, making use of internal *Xho* I sites, from the 3′ ends of the coding sequences and the downstream noncoding regions (shown by arrow for PM255 in Fig. 1). The resulting fragments, encompassing nucleotides 971–1354 (PM81) and 1003–1305 (PM255), were transcribed into biotinylated probes *in vitro* by using T7 RNA polymerase, and the probes were hybridized to size-fractionated, nitrocellulose-blotted RNA obtained from aseptically grown plants representing different stages of development. After incubation with the fragment from PM255, blots were stripped and rehybridized with the PM81-derived probe. Furthermore, microsomal and soluble protein was prepared from plants of the corresponding developmental stages and subjected to $NaDodSO_4$/PAGE, followed by blotting of the proteins onto nitrocellulose and labeling with affinity-purified nitrilase antiserum. This way, mRNA abundance, amount of polypeptide, and intracellular localization could be checked simultaneously

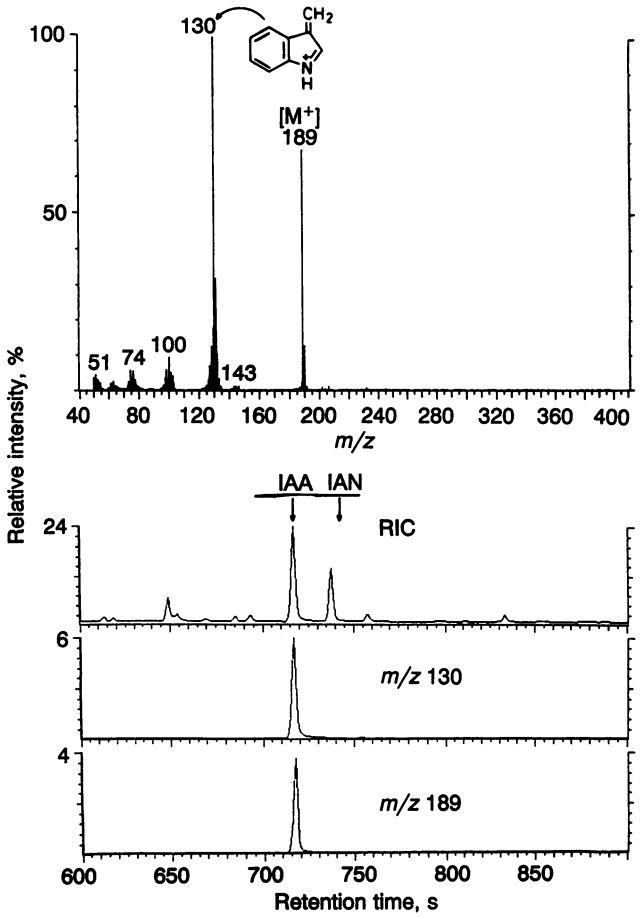

FIG. 4. *A. thaliana* nitrilase II synthesizes IAA from IAN. (*Lower*) Reconstructed total ion current (RIC) as well as single ion traces from a GC-MS analysis of the acidic ethyl acetate phase after separation by HPLC from bacterial host extracts harboring cDNA PM255 and methylation. (*Upper*) Full-scan electron impact (70 eV) of material eluting at the retention time (717 s) of IAA. Spectrum is identical to that of synthetic IAA. Control bacteria do not produce detectable amounts of IAA from IAN. All controls (not shown) and experimental conditions are as detailed in ref. 12. Retention time of IAN, 742 s (absence of *m/z* 130 at this retention time indicates complete removal of IAN substrate during cleaning of samples by HPLC).

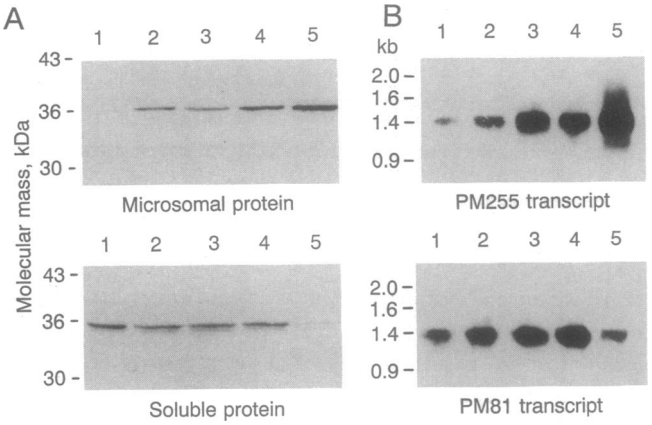

FIG. 5. Nitrilases I and II are differentially expressed during *A. thaliana* development. (*A*) Western blots of membrane-associated and soluble nitrilase from plants at five developmental stages. (*B*) Northern blot analysis of the same developmental stages using sequence-specific probes. Lanes: 1, young, vegetative rosettes (2 weeks old); 2, vegetative rosettes (3 weeks old); 3, plants at early bolting stage (4 weeks old); 4, plants at early flowering stage (5–6 weeks old); 5, beginning of silique growth (6–8 weeks old). Same amounts of protein (80 μg per lane) or RNA (2 μg per lane) were loaded in each lane.

(Fig. 5). PM255 mRNA is hardly detectable in young vegetative rosettes, but its steady-state level increases during bolting and flowering to reach maximum abundance in plants at the early fruiting stage. In contrast, PM81 mRNA can be detected at comparable levels during all stages of vegetative development, early bolting, and flowering stage, but it decreases in abundance during fruit set. Strikingly, the amounts of membrane-bound nitrilase correlate very closely with the abundance of PM255 mRNA while the soluble nitrilase parallels PM81 abundance. Thus, there is evidence that nitrilase I encoded by PM81 represents the soluble enzyme, while PM255 encodes the plasma membrane enzyme, nitrilase II, but verification requires purification of the two nitrilases and N-terminal sequencing.

DISCUSSION

From recent investigations under well-defined conditions (e.g., see refs. 3–6), it has become clear that more than one pathway for the biosynthesis of the phytohormone IAA exists in higher plants. Either tryptophan (3, 4) or, in a nontryptophan pathway, an anthranilate-derived, pretryptophan metabolite—probably indole-3-glycerophosphate—may serve as precursors for IAA (5, 6). The work of Thimann and associates (28, 29) has proven that IAN is converted to IAA by the action of nitrilase in the Brassicaceae and members of the Poaceae as well as other, but not all, plant families. Conversion to the acid is held responsible for the strong auxin activity of IAN (29, 30) and, likewise, the auxin activity of phenylacetonitrile (31) may be explained due to conversion to phenylacetic acid, an endogenous auxin in several species (32, 33). IAN is accessible in the Brassicaceae from at least three sources: (*i*) via tryptophan in the indoleacetaldoxime pathway (9), (*ii*) from degradation of the glucosinolate glucobrassicin (10, 34), and (*iii*) as an intermediate in the tryptophan-independent pathway as proven for *A. thaliana* (6). Nitrilase thus catalyzes a central step in auxin biosynthesis from its direct precursor, IAN, and nitrilase will therefore be instrumental to understand IAA biosynthesis and its regulation at the molecular level.

The two nitrilase genes proposed to occur in the *A. thaliana* genome from Southern analyses (12) have now been cloned as cDNAs, showing that both genes are indeed expressed. Both enzymes, nitrilases I and II, convert IAN to IAA (ref. 12; Fig. 4). Together with the different routes to IAN, redundancy of the pathways to IAA seems established in *A. thaliana*, which would explain why auxin-deficient mutants were never found in this (and other) species (35). The occurrence of more than one isoenzyme for such a key step in plant metabolism would allow—through differential mechanisms of gene expression and enzyme regulation—fine tuning of the synthesis of the hormone in adjustment to varying needs, as well as permit the enzyme to exert additional functions in metabolism. Members of the Brassicaceae accumulate different glucosinolates to high levels in seed and fruit tissues. These are being metabolized during germination and early seedling growth, likely to provide a source of auxin, but also sulfur and nitrogen to the germinating seed, an aspect in early development in which nitrilases might also be involved.

Nitrilase I and nitrilase II are similar in primary sequence and enzymatic characteristics, yet they are found in different cellular compartments and are differentially expressed throughout plant development. The primary sequence classifies both enzymes as relatively polar, and putative transmembrane segments are absent from the sequences. Yet, nitrilase II is plasma membrane bound while nitrilase I is

soluble. We searched for consensus sequences for posttranslational modifications with membrane anchors, but none was apparent. Triton X-114 phase partitioning (25) then revealed nitrilase II to partition into the detergent-poor, hydrophilic phase. The enzyme is thus a peripheral membrane protein and association with the plasma membrane through a quaternary complex with other proteins is indicated. Since the primary structures of both nitrilases diverge at their C and N termini, target residues for the plasma membrane association of enzyme II may be located in these domains.

Nitrilase I, the soluble enzyme, dominates throughout vegetative development and decreases only when plants start to mature (fruit development), while isoenzyme II becomes most abundant at this stage. It is present only at low levels during vegetative development (cf. Fig. 5). Within the shoot, enzyme II is most abundant in the siliques, while it could not be detected in the roots. In contrast, the soluble enzyme is present in all tissues including roots in similar abundance (data not shown). The results show differential compartmentation and regulation of auxin biosynthesis in *A. thaliana*. Under the experimental conditions used by Normanly *et al.* (6)—i.e., precursor feeding to ≈2-week-old plantlets—nitrilase I should have been operating predominantly. The homologous, soluble enzyme was also the one studied earlier (28, 29), and it may be the one responsible for the basic auxin supply of the growing plant. Nitrilase II parallels the abundance and distribution of glucosinolates in *A. thaliana* and other Brassicaceae (11, 36–39), suggesting functional connections. The enzyme could be involved in furnishing auxin from glucosinolates during the early stages of seed germination and seedling growth (36).

From a comparison of the two available higher plant nitrilase sequences with the two prokaryotic sequences (26, 27), it becomes evident that nitrilases form a superfamily of enzymes conserved through evolution from bacteria to higher plants. Although the catalytic center cysteine (27) is conserved in all four sequences and occurs in an environment of relatively high sequence consensus, the overall sequence divergence among the prokaryotic and eukaryotic sequences is considerable (cf. Fig. 3), making it unlikely that probes from bacterial sequences can be used to engineer auxin biosynthesis in higher plants. We are therefore studying transgenic plants that express nitrilase in either sense or antisense orientation. Preliminary results on phenotypes of these plants reinforce our conclusion that nitrilase is an essential element in auxin biosynthesis in *A. thaliana*.

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1. Thimann, K. V. (1977) *Hormone Action in the Whole Life of Plants* (Univ. of Mass. Press, Amherst).
2. Marumo, S. (1986) in *Chemistry of Plant Hormones*, ed. Takahashi, N. (CRC, Boca Raton, FL), pp. 9–56.
3. Michalczuk, L., Ribnicky, D. M., Cooke, T. J. & Cohen, J. D. (1992) *Plant Physiol.* **100**, 1346–1353.
4. Bialek, K., Michalczuk, L. & Cohen, J. D. (1992) *Plant Physiol.* **100**, 509–517.
5. Wright, A. D., Sampson, M. B., Neuffer, M. G., Michalczuk, L., Slovin, J. P. & Cohen, J. D. (1991) *Science* **254**, 998–1000.
6. Normanly, J., Cohen, J. D. & Fink, G. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10355–10359.
7. Last, R. L. & Fink, G. R. (1988) *Science* **240**, 305–310.
8. Last, R. L., Bissinger, P. H., Mahoney, D. J., Radwanski, E. R. & Fink, G. R. (1991) *Plant Cell* **3**, 345–358.
9. Ludwig-Müller, J. & Hilgenberg, W. (1988) *Physiol. Plant.* **74**, 240–250.
10. Butcher, D. N., El-Tigani, S. & Ingram, D. S. (1974) *Physiol. Plant Pathol.* **4**, 127–140.
11. Hogge, L. R., Reed, D. W., Underhill, E. W. & Haughn, G. W. (1988) *J. Chromatogr. Sci.* **26**, 551–556.
12. Bartling, D., Seedorf, M., Mithöfer, A. & Weiler, E. W. (1992) *Eur. J. Biochem.* **205**, 417–424.
13. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15**, 473–479.
14. Larsson, C. (1985) in *Cell Components*, eds. Haskins, H. F. & Jackson, J. F. (Springer, Berlin), Vol. 1, pp. 85–104.
15. Meyer, C., Feyerabend, M. & Weiler, E. W. (1989) *Plant Physiol.* **89**, 692–699.
16. Blum, W., Key, G. & Weiler, E. W. (1988) *Physiol. Plant.* **72**, 279–287.
17. Raymond, C. K., O'Hara, P. J., Eichinger, G., Rothman, J. H. & Stevens, T. H. (1990) *J. Cell Biol.* **111**, 877–892.
18. Brent, R. & Ptashne, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4204–4208.
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmann, J. G., Smith, J. A. & Struhl, K., eds. (1987) *Current Protocols in Molecular Biology* (Wiley, New York).
20. Mertens, R., Eberle, J., Arnscheidt, A., Ledebur, A. & Weiler, E. W. (1985) *Planta* **166**, 389–393.
21. Höhfeld, J., Veenhuis, M. & Kunau, W. H. (1991) *J. Cell Biol.* **114**, 1167–1178.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
23. Higgins, D. G. & Scharp, P. M. (1988) *Comput. Appl. Biosci.* **5**, 151–153.
24. Bartlett, S. G., Grossman, A. R. & Chua, N. H. (1982) in *Methods in Chloroplast Molecular Biology*, eds. Edelman, M., Hallick, R. & Chua, N. H. (Elsevier, Amsterdam), pp. 1081–1091.
25. Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607.
26. Stalker, D. M., Malyi, L. D. & McBride, K. E. (1988) *J. Biol. Chem.* **263**, 6310–6314.
27. Kobayashi, M., Izui, H., Nagasawa, T. & Yamada, H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 247–251.
28. Thimann, K. V. (1958) *Nature (London)* **181**, 1466–1467.
29. Thimann, K. V. & Mahavedan, S. (1964) *Arch. Biochem. Biophys.* **105**, 133–141.
30. Bentley, J. A. & Housley, S. (1952) *J. Exp. Bot.* **3**, 393–405.
31. Wheeler, A. W. (1977) *Ann. Bot.* **41**, 867–872.
32. Okamoto, T., Isogai, Y. & Koizumi, T. (1967) *Chem. Pharm. Bull.* **15**, 159–163.
33. Wightman, F. (1973) *Biochem. Soc. Symp.* **38**, 247–275.
34. Cole, R. A. (1976) *Phytochemistry* **15**, 759–762.
35. Klee, H. & Estelle, M. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 529–551.
36. Elliott, M. C. & Stowe, B. B. (1971) *Plant Physiol.* **48**, 498–503.
37. Bergmann, F. (1970) *Z. Pflanzenphysiol.* **62**, 362–375.
38. Daxenbichler, M. E., Spencer, G. F., Carlson, D. G., Rose, G. B., Brinker, A. M. & Powell, R. G. (1991) *Phytochemistry* **30**, 2623–2639.
39. McClellan, D., Kott, L., Beversdorf, W. & Ellis, B. E. (1993) *J. Plant Physiol.* **141**, 153–159.