Arabidopsis Mutants Resistant to the Auxin Effects of Indole-3-Acetonitrile Are Defective in the Nitrilase Encoded by the NIT1 Gene

Jennifer Normanly, Paula Grisafi, Gerald R. Fink, and Bonnie Bartel

Indole-3-acetonitrile (IAN) is a candidate precursor of the plant growth hormone indole-3-acetic acid (IAA). We demonstrated that IAN has auxinlike effects on Arabidopsis seedlings and that exogenous IAN is converted to IAA in vivo. We isolated mutants with reduced sensitivity to IAN that remained sensitive to IAA. These mutants were recessive and fell into a single complementation group that mapped to chromosome 3, within 0.5 centimorgans of a cluster of three nitrilase-encoding genes, NIT1, NIT2, and NIT3. Each of the three mutants contained a single base change in the coding region of the NIT1 gene, and the expression pattern of NIT1 is consistent with the IAN insensitivity observed in the nit1 mutant alleles. The half-life of IAN and levels of IAA and IAN were unchanged in the nit1 mutant, confirming that Arabidopsis has other functional nitrilases. Overexpressing NIT2 in transgenic Arabidopsis caused increased sensitivity to IAN and faster turnover of exogenous IAN in vivo.

INTRODUCTION

Several pathways have been proposed to account for indole-3-acetic acid (IAA) biosynthesis; however, none of these has been shown definitively to be responsible for the synthesis of the hormone in vivo (Normanly et al., 1995; Bartel, 1997). Plant mutants disrupted in IAA biosynthesis should help to illuminate these pathways, because auxotrophic mutants have been invaluable in elucidating a wide variety of biosynthetic pathways, from amino acid biosynthesis in microbes (Jones and Fink, 1982) to gibberellic biosynthesis in higher plants (Reid, 1993; Reid and Howell, 1995). Despite considerable effort, researchers have not identified IAA biosynthetic mutants (Blonstein et al., 1988; Oetiker et al., 1990; Fracheboud and King, 1991), perhaps because IAA is essential for growth and because plants appear to have multiple pathways for IAA biosynthesis (Michalczuk et al., 1992).

Indole-3-acetonitrile (IAN) has been proposed as an IAA precursor in certain higher plants (reviewed in Schneider and Wightman, 1974; Sembdner et al., 1981; Nonhebel et al., 1993). IAN was first purified from cabbage (Henbest et al., 1953) and is present in Arabidopsis at levels comparable to that of total IAA (Normanly et al., 1993; Ilic et al., 1996). Consistent with the possibility that IAN serves as a precursor to IAA, Arabidopsis mutants blocked in either of the last two steps of tryptophan biosynthesis accumulate both total IAA and IAN (Normanly et al., 1993). Extracts from a variety of plant families, including the Cruciferae, Gramineae, and Musaceae, hydrolyze IAN to IAA (Thimann and Mahadevan, 1964). Genes encoding nitrilase enzymes have been cloned from Arabidopsis (Bartling et al., 1992, 1994; Bartel and Fink, 1994; Hillebrand et al., 1996; Zhou et al., 1996a, 1996b), and similar genes are present in tobacco (Tsunoda and Yamaguchi, 1995), Chinese cabbage (Bischoff et al., 1995), and rice (http://www.staff.or.jp/).

IAA may also be derived from IAN in several plant-associated microbes. These organisms convert IAN to IAA by using either a nitrilase or the sequential action of a nitrile hydratase and an amidase. In Alcaligenes faecalis JM3, an IAN-specific nitrilase has been purified and the corresponding gene cloned (Kobayashi et al., 1993). This enzyme is ~30% identical to the Arabidopsis nitrilase enzymes (Bartel and Fink, 1994). In contrast, the plant pathogen Agrobacterium and several species of the plant symbiont Rhizobium have been shown to convert IAN to IAA through the nitrile hydratase pathway (Kobayashi et al., 1995).

We are exploring the role of nitrilase genes in IAA biosynthesis in the model crucifer Arabidopsis. Each enzyme encoded by the four Arabidopsis nitrilase genes (NIT1, NIT2, NIT3, and NIT4) can hydrolyze IAN to IAA when expressed in
Escherichia coli (Bartling et al., 1992, 1994; Bartel and Fink, 1994). These genes are differentially expressed during development, and the NIT2 gene is specifically induced in response to bacterial pathogen infiltration (Bartel and Fink, 1994). NIT1 is the most highly expressed nitrilase gene, as determined by RNA gel blot analysis, expression of promoter-reporter gene fusions in transgenic plants, and cDNA abundance (Bartel and Fink, 1994). Here, we describe the isolation and analysis of point mutations in the NIT1 gene and use these nitl mutants, along with transgenic plants overexpressing the various nitrilase cDNAs, to investigate the possibility that IAN is an intermediate in IAA biosynthesis.

RESULTS

IAN Has Auxinlike Effects on Germinating Arabidopsis Seedlings

Arabidopsis seedlings germinated on IAN displayed several effects suggestive of but distinct from those caused by exogenous IAA. The most dramatic alteration was seen at the base of the hypocotyl, where the connection between the stele and the cortical and epidermal cells disintegrated and adventitious lateral root primordia developed on the hypocotyl. In addition, IAN inhibited root elongation and caused epinastic cotyledons, as shown in Figure 1B. This induction of lateral root initiation and suppression of root elongation was also observed with IAA (Figure 1C), although the magnitude of the effects was different. In particular, exogenous IAN was more effective in producing auxin effects in the shoot, whereas roots were more sensitive to exogenous IAA. These differences might be due to differences in the transport or metabolism of the two molecules.

Consistent with the possibility that IAN is acting as an auxin in Arabidopsis, IAN effects were decreased in several mutants isolated on the basis of their resistance to various natural and synthetic auxins. In particular, the aux1 (Pickett et al., 1990), axr1 (Lincoln et al., 1990), and axr2 (Wilson et al., 1990) mutants were resistant to IAN (Figure 1B and data not shown). The axr1 mutant was resistant to both the hypocotyl disintegration and the inhibition of root elongation caused by IAN (Figure 1B). However, the aux1 mutant was resistant to the root elongation inhibition but remained sensitive to the IAN-induced hypocotyl disintegration (Figure 1B). This IAN sensitivity in the aux1 shoot is consistent with other phenotypes of the aux1 mutant, which is disrupted in auxin perception only in the root (Pickett et al., 1990), whereas the axr1 mutation has a pleiotropic phenotype with defects in both root and shoot tissues (Lincoln et al., 1990). In contrast, the lir1 mutant, which is resistant to certain IAA-amino acid conjugates but sensitive to free IAA (Bartel and Fink, 1995), was no different from the wild type in its sensitivity to IAN (data not shown). Each of the mutants showed normal root development in the absence of added IAA or IAN (Figure 1A).

The auxin effects of IAN may result from nitrilase-mediated hydrolysis, and the location of these effects might reflect the tissue-specific expression of the various isozymes. Arabidopsis contains four nitrilase genes (Bartling et al., 1992, 1994; Bartel and Fink, 1994) that are differentially expressed during development and in response to environmental stimuli (Bartel and Fink, 1994). Transgenic plants carrying a promoter-reporter gene fusion show that the NIT1 gene is strongly expressed at the base of the hypocotyl (Bartel and Fink, 1994). This region is particularly sensitive to the effects of exogenous IAN (Figure 1B). The coincident sensitivity to IAN and the expression pattern of NIT1 suggest that sensitivity to IAN results from hydrolysis to IAA by the NIT1 enzyme.
Mutants Insensitive to IAN Map to the NIT1 Locus

To isolate mutants with disrupted nitrilase function, we screened for mutants resistant to the auxin effects of IAN but that retain IAA sensitivity. We isolated three such mutants from the progeny of seeds that had been mutagenized with ethyl methanesulfonate (see Methods). These mutants were recessive to the wild type and fell into a single complementation group, which we subsequently determined was disrupted in the NIT1 locus (see below). When germinated on concentrations of IAN ranging from 10 to 80 μM, each of the three nit1 mutant alleles showed increased root elongation and increased hypocotyl integrity compared with that of the wild type (Figure 1B and data not shown). Quantification of this phenotype is shown in Figure 2. After 8 days on 30 μM IAN (a concentration that inhibits wild-type root elongation by >80%), the nit1 mutant showed three- to fourfold increased root elongation compared with that of the wild type (Figures 1B and 2). In contrast, nit1 root elongation did not differ from that of the wild type when germinated on plates containing 0.6 to 3 μM IAA (Figures 1C and 2 and data not shown), showing that IAA sensitivity was not altered in the mutant.

We mapped the nit1 mutant (see Methods) to the region of chromosome 3 that contains a cluster of three nitrilase genes, NIT1, NIT2, and NIT3, within 12 kb of each other (Bartel and Fink, 1994). This mapping data, along with the colocalization of IAN effects and NIT1 expression discussed previously, suggested that the IAN-resistant mutants might be disrupted in the NIT1 gene. To test this directly, we sequenced the NIT1 gene from each of the nit1 mutant alleles and compared these sequences with that of the wild-type (ecotype Columbia [Col-0] sequence (Zhou et al., 1996a). Each mutant allele had a single base substitution that changed an amino acid (nit1-1 and nit1-2) or prematurely terminated the NIT1 polypeptide (nit1-3). As shown in Figure 3A, all three of the nit1 mutant alleles were located in the fourth exon. The changes in the nit1-1 and nit1-2 alleles both substitute charged residues (aspartate or arginine) for glycine residues that are invariant not only in all four Arabidopsis genes (Figure 3B) but also in a similar gene from Chinese cabbage (Bischoff et al., 1995), two related tobacco genes (Tsunoda and Yamaguchi, 1995), and similar genes from the microbes A. faecalis JM3 (Kobayashi et al., 1993), Rhodococcus rhodochrous J1 (Kobayashi et al., 1992b), and R. rhodochrous K22 (Kobayashi et al., 1992a).

Overexpression of NIT Genes in Arabidopsis

Each of the four Arabidopsis nitrilase enzymes is capable of hydrolyzing IAN to IAA when expressed in E. coli (Bartling et al., 1992, 1994; Bartel and Fink, 1994), suggesting a role for nitrilases in plant IAA biosynthesis. The isolation of IAN-resistant nit1 mutants described here demonstrates that the NIT1 enzyme is capable of performing this hydrolysis in vivo as well. To test whether any of the other Arabidopsis nitrilase isozymes can hydrolyze IAN in vivo, we made transgenic plants expressing the NIT1, NIT2, NIT3, and NIT4 cDNAs from the highly expressed cauliflower mosaic virus 35S promoter. RNA gel blot analysis with gene-specific probes was used to select the lines showing the greatest increase in NIT message. The results are shown in Figure 4. These overexpressing lines were tested for their sensitivity to IAN and IAA. Figure 5 shows that overexpression of the NIT2 cDNA from the 35S promoter dramatically increased sensitivity to IAN compared with the wild type, whereas all of the lines retained similar sensitivity to IAA. This hypersensitivity suggests that NIT2, like NIT1, is capable of hydrolyzing IAN in vivo.

We also determined whether the various 35S–NIT constructs could suppress the IAN-resistant phenotype of the nit1 mutant. We crossed the transgenic lines expressing the NIT cDNAs from the 35S promoter (Figures 4 and 5) to the nit1-1 mutant, and we plated the F2 or F3 plants homozygous for the nit1-1 mutation and the transgene (see Methods) on plates containing IAN. The homozygous nit1 mutant overexpressing NIT2 from the 35S promoter was as sensitive to IAN (data not shown) as the 35S–NIT2 construct in a

![Figure 2](image-url). Root Elongation of nit1, aux1, axr1, and Wild-Type Seedlings on IAN and IAA.

Eight-day-old seedlings grown as given in the legend to Figure 1 were removed from the agar, and the length of the longest root was recorded. At least nine seedlings of each genotype were measured for each condition. Shown is the percentage of elongation for either IAN or IAA compared with that of the PNS control. Error bars indicate the standard deviation of the mean.
Figure 3. Mutations in the NIT1 Gene in Three Mutant Alleles.

(A) Genomic structure of the NIT1 locus. Introns are indicated with thin lines between black rectangles (exons). The position of the active site cysteine is indicated with an asterisk.

(B) Alignment of the region encoded by exon 4 in the NIT1 gene, with corresponding regions from other plant and microbial nitrilase genes showing the positions of the mutations found in each of the three nit1 mutant alleles. The nit1-1 mutation changes a GGT (Gly-228) codon to GAT (D, Asp), creating an Mbol restriction site; the nit1-2 mutation changes a GCA (Gly-277) codon to AGA (R, Arg); and the nit1-3 mutation changes a TGG (Trp-255) codon to TAG (stop). Sequences were aligned with the Megalign program (DNAStar, Madison, WI), using the Clustal method. Dashes indicate gaps introduced to maximize alignment, and residues identical to the NIT1 sequence are boxed. Sequences shown are NIT1, NIT2, NIT3, and NIT4 from Arabidopsis (At.), a nitrilase from Brassica rapa (Br.), nitrilases from Rhodochrous (R.r.) strains J1 (Kobayashi et al., 1992b) and K22 (Kobayashi et al., 1992a), and a nitrilase from A. faecalis (Af.) JM3 (Kobayashi et al., 1993).

Figure 4. RNA Gel Blot Analysis of NIT Genes in Transgenic Plants.

Eight micrograms of total RNA prepared from 19-day-old seedlings was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with gene-specific probes NIT1 to NIT4 derived from untranslated regions of the NIT transcripts, as described previously (Bartel and Fink, 1994). Equal loading was confirmed by observation of ethidium bromide–stained rRNAs in each lane. No-0, Nossen; 35SNIT, 35S–NIT2.

Steady State IAN and IAA Levels in nit1 and 35S–NIT2 Plants

Given the IAN resistance of the nit1 mutants (Figure 2) and the increased IAN sensitivity of the 35S–NIT2 line (Figure 5), we quantified free IAA, total IAA (free IAA plus amide- and ester-linked conjugates of IAA), and IAN by isotope dilution gas chromatography–selective ion monitoring–mass spectrometry (GC-SIM-MS) analysis (Normanly et al., 1993; Ilic et al., 1996) in these lines. The nit1-3 allele was selected for this analysis because it showed the most dramatic IAN resistance of the three alleles (Figure 2) and because the lesion in this strain was a premature stop codon (Figure 3B).

As shown in Table 1, steady state levels of free IAA, total IAA, and IAN did not differ from that of the wild type in the nit1-3 mutant. Similarly, the steady state levels of these compounds in 7-, 8-, 13-, or 14-day-old transgenic seedlings overexpressing the various NIT genes from the 35S wild-type background (Figure 5 and data not shown), indicating that NIT2 overexpression is able to suppress the nit1 mutation fully.
promoter did not differ dramatically from those in control lines (Table 1 and data not shown).

**IAN Metabolism in nit1 and 35S–NIT2 Plants**

To explore further IAN metabolism in these lines, we wished to follow the fate of labeled IAN, determining its half-life and conversion to IAA. Several features of the experimental design are critical for valid measurements. Exogenous $^{13}$C$_1$-IAN must be applied in amounts that label the IAN pool but only minimally alter the steady state level of IAN. In addition, steady state IAN levels must remain constant over the time period in which the half-life measurements are made.

We found that a 1-hr pulse of 60 µM $^{13}$C$_1$-IAN administered to 7.5-day-old wild-type seedlings resulted in 30 to 50% labeling of the IAN pool. To determine whether 60 µM IAN perturbed endogenous IAN metabolism, we conducted mock pulse-labeling experiments in which 7.5-day-old seedlings were manipulated in the same manner as in the pulse-labeling experiments, except that either no IAN was present (mock pulse) or 60 µM unlabeled IAN was added (cold pulse). In duplicate experiments, total IAN levels varied by <12% from 1 to 3 hr after the mock pulse. Adding 60 µM IAN during the cold pulse increased IAN levels twofold. This doubling is expected for 50% labeling; therefore, we concluded that exposing the seedlings to 60 µM IAN did not drastically alter endogenous IAN metabolism. (Similar attempts to establish labeling conditions in Arabidopsis for the measurement of IAA turnover that do not substantially perturb IAA metabolism have been unsuccessful [J. Normanly, unpublished data].)

Lastly, improper storage of IAN can result in its nonenzymatic conversion to IAA. Because endogenous IAN levels are at least two orders of magnitude higher than free IAA levels are (Normanly et al., 1993; Table 1), any $^{13}$C$_1$-IAA contamination of the $^{13}$C$_1$-IAN used would artificially elevate the observed incorporation of $^{13}$C, label into IAA. We used HPLC to determine that our $^{13}$C$_1$-IAN contained <0.07% $^{13}$C$_1$-IAA. This would result in at most 42 nM $^{13}$C$_1$-IAA being present during a pulse with 60 µM $^{13}$C$_1$-IAN. In a separate experiment performed in quadruplicate, we determined that a 1-hr pulse with 42 nM $^{13}$C$_1$-IAA resulted in 7% labeling of the free IAA pool. Therefore, 7% was determined to be the maximum background in our labeling experiments described below.

We then directly tested the ability of the nit1 mutant and the 3SS–NIT2 line to metabolize IAN. Seedlings were pulse-labeled with $^{13}$C$_1$-IAN, and the incorporation of label from $^{13}$C$_1$-IAN into IAA was determined by GC-SIM-MS. In addition, we determined the rate at which IAN was metabolized (IAN half-life) in these lines. Adding an internal standard ($^{13}$C$_3$-IAA) upon extraction enabled the determination of free IAA levels.

As shown in Table 2, all of the tested lines hydrolyzed exogenous $^{13}$C$_1$-IAN to $^{13}$C$_1$-IAA, directly demonstrating that Arabidopsis can hydrolyze IAN in vivo. The half-life of IAN in the 3SS–NIT2 line was twofold shorter than was the untransformed control, and the levels of free IAA ($^{13}$C$_1$-labeled plus unlabeled) increased almost fourfold by 1 hr after the $^{13}$C$_1$-IAN

![Figure 5. Root Elongation of NIT Overexpressing and Wild-Type Seedlings on IAN and IAA.](image)

Root elongation of 8-day-old seedlings was quantified as given in the legend to Figure 2, except that 10 µM IAN was used to allow detection of increased sensitivity.

<table>
<thead>
<tr>
<th>Plants</th>
<th>IAN</th>
<th>Free IAA</th>
<th>Total IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>637 ± 108</td>
<td>7 ± 1</td>
<td>1542 ± 150</td>
</tr>
<tr>
<td>nit1-3 (Col-0)</td>
<td>579 ± 20</td>
<td>12 ± 1</td>
<td>1360 ± 10</td>
</tr>
<tr>
<td>Wild type (No-0)</td>
<td>467 ± 10</td>
<td>5 ± 0.3</td>
<td>1455 ± 23</td>
</tr>
<tr>
<td>3SS–NIT2 (No-0)</td>
<td>845 ± 101</td>
<td>8 ± 1</td>
<td>2651 ± 146</td>
</tr>
</tbody>
</table>

*Values are given in nanograms per gram fresh weight of tissue, presented as the mean ± SE from three independent samples, except for free IAA in 3SS–NIT2 and total IAA in Col-0, in which case the sample sizes were two and the values are presented as the mean plus or minus the average deviation from the mean.*
pulse (Table 2). This alteration in IAN metabolism is consistent with the observed increase in sensitivity to IAN in the 35S-NIT2 line (Figure 5).

In contrast, the half-life of IAN in the nit1-3 mutant did not differ significantly from that of the wild type (Table 2). The levels of 13C1-IAA formed in the nit1-3 mutant during the pulse-labeling experiments were also similar to those of the wild type, possibly because of compensatory activity of other nitrilases. However, in two of three nit1-3 samples, the levels of endogenous unlabeled IAA were radically altered (10- to 27-fold above normal) by the 13C1-IAN pulse but only briefly, indicating that in this mutant, IAA metabolism is altered in response to exogenous IAN.

**DISCUSSION**

It is likely that several pathways for IAA biosynthesis coexist within a given plant, as has been demonstrated for carrot (Michalczuk et al., 1992), maize (Wright et al., 1991; Koshiba et al., 1995), and Arabidopsis (Normanly et al., 1993; J. Normanly, unpublished data), all of which show both tryptophan-dependent and tryptophan-independent pathways of IAA biosynthesis. The situation might be further complicated by the existence of multiple genes encoding isozymes of IAA biosynthetic enzymes, as is the case for numerous biosynthetic pathways in Arabidopsis (Lam et al., 1995; Radwanski and Last, 1995). Although this potential redundancy at the level of both pathways and isozymes complicates biochemical analysis, it may provide plants with the precise control necessary for IAA homeostasis.

The nature of the molecular defect of the IAN-resistant nit1 mutants described here implies that IAN exerts its auxinlike effects through its hydrolysis to IAA and that this hydrolysis can be performed in vivo by the product of the NIT1 gene. The increased sensitivity to IAN of plants overexpressing the NIT2 gene and the observation that NIT2 overexpression can suppress the nit1 defect suggest that the NIT2 enzyme is also capable of hydrolyzing IAN to IAA in vivo. These results are consistent with the observation that ectopic expression of the Arabidopsis NIT2 gene in transgenic tobacco results in plants with increased sensitivity to exogenous IAN (Schmidt et al., 1996).

The observation that the 35S-NIT1, 35S-NIT3, and 35S-NIT4 lines did not display increased sensitivity to IAN (Figure 5) or suppress the nit1 mutant phenotype on IAN (data not shown) despite an increase in message accumulation (Figure 4) may indicate that these lines are not overexpressing protein because of post-transcriptional regulation. It is also possible that the NIT3 and NIT4 enzymes, which can hydrolyze IAN to IAA in vitro (Bartel and Fink, 1994), do not do so in vivo.

The data from 13C1-IAN labeling studies directly demonstrate that Arabidopsis seedlings can hydrolyze IAN to IAA in vivo. In all tested lines, 13C1-labeled IAN was hydrolyzed to 13C1-labeled IAA (Table 2). The half-life of IAN was two times longer and the percentage of incorporation of 13C from IAN into IAA was approximately fourfold higher in the 35S-NIT2 line than in the untransformed control. Thus, when the NIT2 enzyme was overexpressed in vivo, it hydrolyzed 13C1-IAA to IAA. These results agree with the observation that ectopic expression of the Arabidopsis NIT2 gene confers the ability to hydrolyze exogenous 13C1-IAA to 13C1-IAA (Schmidt et al., 1996).

Although nitrilase activity in extracts from the transgenic 35S-NIT2 line was elevated (Jutta Ludwig-Müller, personal communication) and the level of 13C1-labeled free IAA recovered after a 13C1-IAN pulse was increased in the 35S-NIT2 line compared with that of the wild type (Table 2), steady state levels of IAN and unlabeled free IAA were not dramatically altered in this line (Tables 1 and 2). A possible explanation, supported by the data from pulse-labeling studies, is that endogenous IAN may be limiting or not accessible to

### Table 2. IAN Half-Life and Incorporation of Label from 13C1-IAA into IAA in 7.5-Day-Old Wild-Type, nit1-3, and Transgenic 35S-NIT2 Seedlings

<table>
<thead>
<tr>
<th>Plants</th>
<th>13C1-IAN (%)</th>
<th>IAN Half-Life (hr)</th>
<th>Free 13C1-IAA (%)</th>
<th>Free IAA (ng/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t1</td>
<td>t2</td>
<td>t1</td>
<td>t1</td>
</tr>
<tr>
<td>Wild type (Col-0)</td>
<td>28 ± 2</td>
<td>9 ± 2</td>
<td>1.5 ± 0.5</td>
<td>49 ± 4</td>
</tr>
<tr>
<td></td>
<td>35S-NIT2 (Col-0)</td>
<td>23 ± 1</td>
<td>10 ± 2</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Wild type (No-0)</td>
<td>26 ± 8</td>
<td>20 ± 8</td>
<td>3.5 ± 1</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>35S-NIT2 (No-0)</td>
<td>32 ± 0.4</td>
<td>14 ± 0.8</td>
<td>1.8 ± 0.2</td>
<td>86 ± 4</td>
</tr>
<tr>
<td></td>
<td>35S-NIT4 (Col-0)</td>
<td>81 ± 4</td>
<td>10 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td></td>
<td>28 ± 2</td>
<td>9 ± 2</td>
<td>1.5 ± 0.5</td>
<td>49 ± 4</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>35S-NIT4 (Col-0)</td>
<td>81 ± 4</td>
<td>10 ± 3</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

aAnalysis was performed in triplicate for Col-0 and nit1-3 (data presented as the mean ±SE) and in duplicate for No-0 and 35S-NIT2 (data presented as the mean plus or minus average deviation). For Col-0 and nit1-3: t1, 1 hr; t2, 3 hr; for No-0 and 35S-NIT2: t1, 1 hr; t2, 2.5 hr.

bThe percentage of 13C1-IAA was determined for each sample by dividing the abundance of ions with an m/z of 229 (corrected for natural abundance of carbon-13 and nitrogen-15, as described in Methods) by the sum of abundances for ions with an m/z of 228 and an m/z of 229.

cThe percentage of free 13C1-IAA was determined for each sample by dividing the nanograms per gram fresh weight of tissue (ng/g FW) value for 13C1-IAA at t1 by the sum of nanogram per gram values for 13C1-IAA and unlabeled IAA.
the overproduced nitrilase. In the 35S-NIT2 line after a 1-hr pulse with 13C-IAN and a 1-hr chase, the total cellular IAN pool was 32% labeled with carbon-13, whereas the IAA pool was 86% carbon-13 labeled. If endogenous IAN and 13C-IAN were both readily utilized by the overproduced nitrilase, then incorporation of label into IAA should be the same or less than incorporation of label into IAN. The most likely explanation for a precursor (IAN) being less enriched than the product (IAA) would entail separate pools of IAN, with the labeled pool being preferentially used as a precursor.

If compartmentation of IAN plays a role in regulation of IAA biosynthesis, then overproduction of NIT2 may not result in increased hydrolysis of endogenous, unlabeled IAN. Biochemical fractionation indicates that NIT1 is a soluble enzyme and that NIT2 is membrane associated (Bartling et al., 1994); however, the subcellular localization of IAN is not known, and we have not localized the overexpressed NIT2 enzyme. IAA has been localized both to the cytosol and to chloroplasts (Sitbon et al., 1993), and the tryptophan biosynthetic enzymes (the source of IAA precursors) have been localized to the chloroplast (Zhao and Last, 1995). Determination of the subcellular location of IAN would be helpful in assessing the degree to which IAN participates in IAA biosynthesis under normal conditions.

Steady state levels of IAA and IAN, the half-life of IAN, and incorporation of carbon-13 from IAN to IAA were not dramatically altered in the nit1-3 mutant (Tables 1 and 2). Given the redundancy of nitrilases in Arabidopsis, it is possible that IAA levels and IAN metabolism are maintained at wild-type levels by the remaining functional nitrilases and other IAA biosynthetic pathways. In addition, subtle differences in IAN or IAA metabolism between the wild-type and nit1 mutant strains may be masked by the necessity of performing biochemical analysis on entire seedlings, whereas a mutant phenotype can reflect local or tissue-specific differences in metabolism. In two of three nit1-3 samples, the endogenous, unlabeled IAA levels were significantly higher (10- to 27-fold) than those of the wild type after a pulse with 13C-IAN, returning to normal levels within 2 hr (Table 2). These observations suggest that IAA metabolism in response to exogenous IAN is altered in the mutant, perhaps contributing to its IAN resistance. IAA levels appeared normal in one of the nit1-3 samples, which we attribute to inadvertent variations in environmental parameters.

Our results provide another example in which reductancy at the level of functionally similar genes does not preclude the isolation of conventional mutants. This was seen previously in the case of tryptophan biosynthetic genes, in which recessive mutants in genes encoding biosynthetic enzymes were isolated by selecting for resistance to toxic intermediates (Last and Fink, 1988; Last et al., 1991; Radwanski et al., 1996). As expected, when the targets were members of gene families, these mutations tended to occur in the genes encoding the most highly expressed isozymes (Last et al., 1991; Niyogi et al., 1993). It is likely that the strong expression of the NIT1 gene at the base of the hypocotyl and the relative absence of expression of the other NIT genes in this tissue (Bartel and Fink, 1994) account for our ability to select for mutations in this gene. However, the observations that the nit1 mutants do not have dramatic morphological phenotypes in the absence of IAN and are not severely disrupted in IAN metabolism suggest that if nitrilases are involved in IAA biosynthesis in Arabidopsis, at least one of the three NIT genes that remain active in the nit1 mutant is able to compensate for its function in supplying IAA to the plant. Alternatively, other pathways of IAA biosynthesis might be induced in the absence of a functional NIT1 gene. Ongoing efforts to identify mutations in the other NIT genes should resolve these possibilities.

METHODS

Arabidopsis thaliana Strains and Growth Conditions

Arabidopsis ecotypes Columbia (Col-0), Landsberg erecta (Ler), and Nossen (No-0) were grown in soil (Metromix 200, Scotts-Sierra Horticultural Products, Marysville, OH) under continuous illumination at 22 to 25°C. Plants were grown aseptically on plant nutrient medium with 0.5% sucrose solidified with 0.6% agar (PNS; Haughn and Somerville, 1986) or PNS supplemented with 10 to 80 µM indole-3-acetonitrile (IAN; from 100 mM stock in ethanol), 0.5 to 3 µM indole-3-acetic acid (IAA; from 10 mM stock in ethanol), or 20 µg/mL kanamycin (from 25 mg/mL stock in water). Plates were wrapped in gas-permeable surgical tape (3M, St. Paul, MN) and grown under continuous illumination (25 to 45 µE m⁻² sec⁻¹) with yellow long-pass filters to reduce the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990). Plants used for analysis of IAA and IAN were Col-0 (wild type) or homozygous nit1-3 mutant plants that had been backcrossed to Col-0 twice. Transgenic plants were the T₁ or T₂ generation. Plants were grown aseptically as above in the absence of IAN or IAA in 150 × 25 mm Petri plates ( Falcon 1013; Becton Dickinson Labware, Lincoln Park, NJ) at a density of 1000 seeds per plate under continuous illumination (100 µE m⁻² sec⁻¹). Seedlings were harvested at varying times after germination, weighed, frozen in liquid nitrogen, and stored at −80°C.

For IAN turnover studies, seeds were surface sterilized (Last and Fink, 1988), resuspended in molten PNS made with 0.55% agar (ultrapure; U. S. Biochemical), and sown on sterile nylon mesh discs (catalog No. 3-500-49; Tetko, Rochester, NY) that had been placed atop the same medium in 150 × 25 mm Petri plates. Plates were wrapped as given above and incubated at room temperature under continuous illumination (100 µE m⁻² sec⁻¹). After 7.5 days, the seedlings were transferred to Petri plates containing 25 mL of sterile liquid PN medium (PNS lacking ferric EDTA and sucrose) and 60 µM of 13C-IAN (Ilic et al., 1996). Transfer was accomplished by gently lifting the nylon mesh discs from the agar by using sterile forceps and taking care to remove any clumps of solidified media that clung to the roots. Seedlings were incubated under continuous illumination (25 to 45 µE m⁻² sec⁻¹) with yellow long-pass filters for 1 hr. The seedlings were then washed extensively with sterile water to remove excess label and transferred to fresh liquid PN medium lacking both 13C-IAN and ferric EDTA and incubated for 2.5 to 3 hr. Samples were harvested at 1 and 2.5 or 3 hr after transfer to fresh medium by gently pulling the seedlings from the nylon mesh with forceps, blotting...
excess moisture with Kimwipes, weighing, and freezing in liquid nitrogen. Samples were stored at −80°C.

**Mutant Screen**

The nit1 mutant alleles were isolated as follows. Col-0 seeds (10,000) were mutagenized with 0.24% (v/v) ethyl methanesulfonate for 16 hr at room temperature. M₁ seeds were washed extensively with water and sown in 16 pools of ~625 seeds and allowed to self-fertilize. Of the resulting M₂ seeds, 40,000 were surface sterilized (Last and Fink, 1988) and spread on 150 × 25 mm Petri plates containing 100 mL of PNS supplemented with 80 μM IAN at a density of ~500 seeds per plate. After 2 weeks, putative mutants with increased root length and hypocotyl integrity were transferred to soil and allowed to set seeds. The resultant M₃ seeds were screened for resistance to 50 μM IAN or 3 μM IAA. Four mutants from three pools were identified that were resistant to IAN and sensitive to IAA. These four mutants were the complement one another and represented three independent nit1 alleles (nit1-2 to nit1-3) based on sequence analysis.

**Generation of Transgenic Plants**

Full-length cDNAs encoding each of the nitrilase cDNAs were cloned downstream of the cauliflower mosaic virus 35S promoter in the vector pBlCaMV (a vector derived from pB1121 [Jefferson et al., 1987] in which the gene encoding β-glucuronidase was replaced by a multiple cloning site [J. Celenza, personal communication]). These constructs were introduced into Agrobacterium tumefaciens LBA4404 by electroporation. The resultant strains were used to construct transgenic plants (ecotype No-0) by using the method of root transformation, as previously described (Valvekens et al., 1988). Seeds from regenerated plants were plated on PNS supplemented with 20 μg/mL kanamycin, and homozygous lines were identified in subsequent generations. RNA was prepared from homozygous plants, and relative levels of nitrilase gene expression were compared using RNA gel blot analysis with gene-specific probes (Bartel and Fink, 1994). One line from each of the four overexpressing constructs showing the highest increase in mRNA abundance for the gene of interest was selected for additional experiments.

**Genetic Analysis**

*nit1* plants were backcrossed to the Col-0 ecotype for phenotypic analysis and outcrossed to the Ler ecotype for genetic mapping. DNA was prepared (Celenza et al., 1995) from nit1 F₂ plants and analyzed by polymerase chain reaction (PCR) with primers that amplify the NIT1-NIT2 intragenic region on chromosome 5 (Bartel and Fink, 1994). These primers detect a length polymorphism between the Col-0 and Ler ecotypes. Two nit1 mutant alleles were analyzed and showed 100% linkage (38 plants for nit1-1 and 66 plants for nit1-2) to the Col-0 ecotype in this analysis, corresponding to a map distance of <0.5 centimorgans.

The nit1-1 mutant was crossed to transgenic plants carrying the NIT1, NIT2, NIT3, or NIT4 cDNAs driven by the cauliflower mosaic virus 3SS promoter. F₂ plants carrying the transgene were selected on plates containing 20 μg/mL kanamycin, and the genotype of these plants at the NIT1 locus was determined by cleavage of a NIT1 PCR product with the enzyme MboI. A new MboI site was generated by the G → A base change of the nit1-1 allele. To avoid DNA from the transgene in this analysis, DNA encompassing exon 3, intron 3, and exon 4 was amplified using primers designed from intron 2 (N1-14: 5'-CTAATTAGGTTGTTGCTCTAG-3') and intron 4 (N1-20: 5'-GATGACTATCAAATGAAGTAGT-3'). PCR conditions were 50 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C. Kanamycin-resistant plants homozygous for the nit1-1 mutation were allowed to self-fertilize and set seeds. F₂ or F₃ families that gave 100% kanamycin-resistant seedlings (indicating that the transgene locus was homozygous) were plated on 50 μM IAN to assay suppression of the IAN-resistant phenotype.

**Sequencing of Mutant NIT1 Alleles**

For sequencing the nit1 mutant alleles, the NIT1 gene was amplified by PCR (using the primers 5'-GCTGATATTGTCTCTGTTGTC-3' and 5'-CTTGGACTCTCATGGTCTGAGT-3') from genomic DNA prepared from each mutant allele. PCR conditions were 40 cycles of 30 sec at 95°C, 30 sec at 56°C, and 3 min at 72°C. The resultant 2.1-kb PCR products were either sequenced directly (Ausubel et al., 1993) or cloned using the pT7Blue T-vector kit (Novagen, Madison, WI). DNA sequencing was with custom oligonucleotide-primed reactions analyzed on an Applied Biosystems (Foster City, CA) automated DNA sequencer by D. Needleman (University of Texas–Houston Medical School Molecular Genetics Core Facility) using dye terminators or by dye deoxy sequencing using Sequenase DNA polymerase, as described by the manufacturer (U.S. Biochemical). Changes found in the mutant alleles were verified by sequencing from an independent PCR reaction.

**Analysis of IAA and IAN**

Up to 2 g of frozen tissue was ground in a mortar that had been chilled with liquid nitrogen. Glass beads (150 to 212 μm; Sigma) were added to aid tissue disruption. The frozen powder was added to cold extraction buffer (35% 0.2 M imidazole, pH 7.0, 65% isopropanol) to which 50 to 400 ng of 13C₆-IAA (Cambridge Isotope Laboratories, Andover, MA) and up to 400 ng of 13C₆-IAA (Ilic et al., 1996) had been added as internal standards. Samples were equilibrated at −20°C for 1 hr and then boiled for 20 min under a stream of nitrogen to destroy myrosinase activity. Samples were centrifuged briefly in a tabletop centrifuge to pellet tissue debris. The supernatant was removed to a new tube, and the pellet was washed with extraction buffer and centrifuged again. This process was repeated three times, the supernatants were pooled, and isopropanol was removed by rotary vacuum. Approximately 100,000 cpm of 3H-IAA (25.4 Ci/mmol; Amersham, Arlington Heights, IL) was added as a radiotracer. IAN, total IAA, and free IAA were isolated as described previously (Normanly et al., 1993), except that larger capacity (1 g; J & W Scientific, Folsom, CA) amino columns were used. For analysis of samples from 13C₆-IAA pulse label experiments, only IAN and free IAA were isolated, and no additional 13C₆-IAA was added upon extraction.

Gas chromatography–selective ion monitoring–mass spectrometry (GC-SIM-MS) was conducted on a Hewlett Packard (Rockville, MD) model 5890 GC coupled to a Hewlett Packard model 5972 MS. For IAA analysis, either a 15-m DB1701 or 30-m DB5MS column (J & W Scientific) was used. The initial temperature was 140°C, ramped at 5°C per min with a helium flow rate of <1 mL/min to a final temperature of 280°C. IAN analysis was performed on either a 30-m DB5MS or 15-m DB5 GC column (J & W Scientific). The initial temperature was 140°C, ramped at 20°C per min with a helium flow rate of <1
two or three times by GC-MS. For determination of total IAA levels, molecular ions with an m/z of 189, 190, and 195 were monitored. For free IAA quantification, quinolinium and molecular ions with an m/z of 130, 136, 189, and 195 were monitored; for free IAA samples resulting from 13C1-IAN pulse labeling experiments, molecular ions with an m/z of 189, 190, and 195 were monitored; for IAN samples, the molecular ions monitored were the same as for IAN quantification.

IAN half-life values were calculated as described previously (Zilversmit et al., 1943), according to the following formula: \( t_{1/2} = \ln 2/k \), where \( k \), the first-order rate constant, is in \( C_C/C_C \times 1/t \). \( C_C \) is the percentage of 13C enrichment of IAN at the first time point (1 hr), \( C_t \) is the 13C enrichment of IAN at the second time point (2.5 or 3 hr), and \( t \) is the time elapsed in hours between \( C_C \) and \( C_t \). The percentage of carbon-13 enrichment for IAN was calculated after dividing the value for m/z 228 by the sum of the values for m/z 228 and m/z 229 after correcting for the natural abundance of carbon-13 and nitrogen-15. The percentage of carbon-13 enrichment of IAA was determined by dividing the value for 13C-IAA (nanograms per gram fresh weight) by the sum of the values for 13C-IAA and unlabeled IAA (nanograms per gram fresh weight).

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J Normanly, P Grisafi, G R Fink and B Bartel

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