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Fast track to the trichome: induction of N-acyl nornicotines precedes nicotine induction in *Nicotiana repanda*

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Abstract. *Nicotiana repanda* Wildenow ex Lehmann acylates nornicotine in its trichomes to produce N-acyl-nornicotine (NacNN) alkaloids which are dramatically more toxic than nicotine is to the nicotine-adapted herbivore, *Manduca sexta*. These NacNNs, like nicotine, were induced by methyl jasmonate (MeJA) and wounding, but the 2-fold increase in NacNN pools was much faster (within 6 h) than the MeJA-induced increase in nornicotine pools (24 h to 4 d), its parent substrate. When $^{15}NO_3^-$ pulse-chase experiments with intact and induced plants were used to follow the incorporation of ^{15}N into alkaloids in different plant parts over the plant's lifetime, it was found that the root nicotine pool was most rapidly labeled, followed by the shoot nornicotine and NacNN pools. After 3 d, 3.12% of ^{15}N acquired was in nicotine (0.93%), nornicotine (0.32%) and NacNNs (1.73%) while only 0.14% was in anabasine. Once NacNNs are externalized to the leaf surface, they are not readily re-distributed within the plant and are lost with senescing leaves. The wound- and MeJA-induced N-acylation of nornicotine is independent of induced changes in nornicotine pools and the rapidity of the response suggests its importance in defense against herbivores.

Key words: Acylnornicotine – Induced defense – Methyl jasmonate – *Nicotiana* (induced defense) – Nicotine – Trichome – Wounding

Many secondary metabolites that are important in the resistance of plants against herbivores and pathogens are rapidly up-regulated after attack (Karban and Baldwin 1997), and nicotine, the potent poison of acetylcholine receptors, is a well-studied example (Baldwin 1999). In a number of *Nicotiana* species, nicotine biosynthesis, which is restricted to the roots, is rapidly induced by herbivore attack, wounding or exogenous applications of the endogenous wound hormone, jasmonic acid (JA) or its methyl ester (MeJA; Baldwin 1988a; Baldwin and Ohnmeiss 1993; Baldwin et al. 1996, 1997; McCloud and Baldwin 1997). In tests with native populations of *N. attenuata*, MeJA-induced increases in nicotine are associated with decreased attack from vertebrate herbivores and higher plant fitness (Baldwin 1998). Some insects, such as the larvae of *Manduca sexta*, specialize on tobacco and have evolved resistance to nicotine (Self and Guthrie 1964).

Nicotiana repanda, like the other members of the *Repandae* section of the genus (*N. stocktonii* and *N. nesophila*), has the ability to acylate nornicotine to produce a novel group of alkaloids – hydroxylated and nonhydroxylated N-acyl-nornicotines (NacNNs). These structures are about 1000-fold more toxic than nicotine is to *Manduca sexta* (Jones et al. 1985; Huesing and Jones 1988; Severson et al. 1988b) and may have resulted from cycles of evolutionary responses between the ancestral plant and insect taxa. Such co-evolutionary interactions are thought to be an important mechanism for the diversification of plant secondary metabolites (Ehrlich and Raven 1964). The hydroxylated and non-hydroxylated NacNNs differ in both branching pattern and chain length (between C_{12} and C_{16}); the N-(3-hydroxy-12-methyl-tridecanoyl) nornicotine is the most abundant structure in *N. repanda* (Severson et al. 1988a). The length of the acyl chain may influence the toxicity of the compound to *M. sexta* since the C_{14} and C_{16} analogues are significantly more toxic than those with shorter chain lengths (Huesing et al. 1989). Jones and co-workers (Zador and Jones 1986; Jones et al. 1987) have demonstrated that nornicotine is N-acylated in the trichomes, and since nicotine is the most abundant alkaloid in roots, stems and phloem, while nornicotine is more prevalent in leaves, NacNNs are likely synthesized from root-produced nicotine that is subsequently

Abbreviations: NacNN = N-acyl-nornicotine; MeJA = methyl jasmonate

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demethylated in the shoot to nornicotine, acylated in the trichomes and rapidly excreted to the leaf surface (Zador and Jones 1986; Jones et al. 1987).

The plant surface is the first interface in the plant-herbivore interaction. It is therefore not surprising that trichomes frequently contain defense metabolites (Levin 1973) or that trichome density increases in leaves produced after herbivore attack (Mauricio et al. 1997; Agrawal 1998). However, since trichome production and maturation is limited to short periods early in leaf development (Huelskamp et al. 1994), they are thought to function primarily as a constitutive defense. Here we demonstrate that a trichome-based response, the production of NacNNs is rapidly induced, and that its induction precedes the induction of its parent substrate, nornicotine.

Plant Growth. Nicotiana repanda Wildenow ex Lehmann seeds (from Oxford Tobacco Research Station, P.O. Box 1555, Oxford, N.C., USA) were germinated and grown in soil for 14 d. Approximately 100 plants were transferred to a 25-L hydroponic chamber containing a complete nutrient solution (Baldwin and Schmelz 1994) and after 5 d, transferred to individual 1-L containers with a no-N hydroponic solution (Ohnmeiss and Baldwin 1994) supplemented with 28 mg N as KNO_3. All plants were grown in walk-in growth rooms (conditions as described by van Dam and Baldwin 1998). After 10 d of growth in the individual chambers, 7.5 mg ^{15}N as ^{15}N-KNO_3 (99.6–99.9 atom%; Isotec, Miamisburg, Ohio, USA) was added to each hydroponic chamber, defining day 0 for each experiment.

Wounding and MeJA treatments. Three experiments were conducted. In the first (Fig. 3), roots were induced by adding 250 μg of MeJA (lot 05310-068; Aldrich) to the hydroponic solution on day 0. Four replicate control and MeJA-induced plants were harvested each day on days 1–5 and on days 13, 22 and 36. In experiment 2 (Fig. 2), plants were induced either with 250 μg of MeJA applied to the roots, or shoots were misted, using a glass perfume sprayer, with 500 μg MeJA suspended in 750 μL distilled water. Control plants were misted with 750 μL of distilled water. Five replicate plants from the control treatment and the two MeJA treatments were harvested at 6, 12, 24 and 48 h after MeJA application. In experiment 3 (data not shown), five replicate plants were either wounded with a fabric pattern wheel, with two rolls on each side of the midrib of four fully expanded leaves (Ohnmeiss et al. 1997), or treated with 100 μg of MeJA applied to the roots, or left undamaged. All plants were harvested on day 4.

Chemical analysis. During the rosette-stage, roots and shoots were harvested and weighed separately. For harvests on days 5, 13, 22 and 36, plants were separated into stems, flowers, seed capsules, and leaves of different stages (young, mature, old, senescing and dead). The NacNNs were extracted by submerging the plant part for 30 s in 10 mL of CH_2Cl_2. Tissues were gently squeezed between the lid and the base of a 10-cm glass petri dish to improve extraction. This extract was evaporated and NacNNs were redissolved in 1 mL acetonitrile containing 50 μg 2,2′-pyridil (lot 12025-047; 97%; Aldrich) as an internal standard. The NacNNs were separated by HPLC (Fig. 1) on a μBondapack column (30 mm long, 3.9 mm i.d.; RP-18 125 Å 10 μm; Waters) with an isocratic mobile phase [eluent 60:40 (v/v) acetonitrile:water buffered with *o*-phosphoric acid and triethylamine to pH 7.3] at a flow rate of 1 mL min^{-1} (Varian 9012Q pump), and detected at UV 254 (UV detector Varian 9050). Relative molar response factors between 2,2′-pyridil and nornicotine were determined and we assumed an equal response for nornicotine and the NacNNs. The individual NacNNs were identified by GC-MS analysis of the corresponding fractions. Nicotine, nornicotine and anabasine contents of roots and shoots were determined by HPLC as described by Baldwin (1988b).

Incorporation of ^{15}N into the alkaloids was determined by GC-MS (HP 5890 II GC/HP5971 MSD; Hewlett Packard). An aliquot of the NacNN HPLC sample was dried and silylated with 50 μL bis(trimethylsilyl)trifluoroacetamide (lot 13806ER; Aldrich) in 100 μL N,N-dimethylformamide (DMF) (lot PR051 62LR; Aldrich) for 30 min at 65 °C. The trimethylsilyl-NacNN derivatives were separated after a 30-s splitless injection (250 °C) on a DB-5 ms GC column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, Calif., USA, the temperature of which was held at 90 °C for 2 min, increased at 20 °C min^{-1} to 280 °C and held at 280 °C for 15 min. The mass spectrometer was scanned with 1.5 scans s^{-1} from m/z 50 to 550. Single and double ^{15}N incorporations were determined from the C_{14}OH-NN peak in the chromatograms. The ion at 175 amu represents the $[\text{nornicotine-CO}]^+$ fragment and exhibited no isotopic discrimination in several test runs. The ion abundances were averaged over the midsection of the GC peak at half peak height. Since no NacNN standards are commercially available, plants grown exclusively with $K^{14}NO_3$ were used as a reference to determine the natural ^{13}C and ^{15}N contributions to these ion fragments. The percentage of ^{15}N incorporation was calculated as:

$$\%\ \text{single inc.} = \{[176/(175+176+177)]_{\text{sample}} - [176/(175+176+177)]_{\text{reference}}\} \cdot 100$$

$$\%\ \text{double inc.} = \{[177/(175+176+177)]_{\text{sample}} - [177/(175+176+177)]_{\text{reference}}\} \cdot 100$$

For determination of ^{15}N incorporation into nicotine, nornicotine and anabasine, 3 mL of the HPLC sample was extracted with 2 mL of CH_2Cl_2 at pH 12 for 12 h. After centrifugation and concentration of the CH_2Cl_2 layer, 1 μL was injected (30 s splitless) on an RTX-5 Amine GC column (30 m × 0.25 mm ×

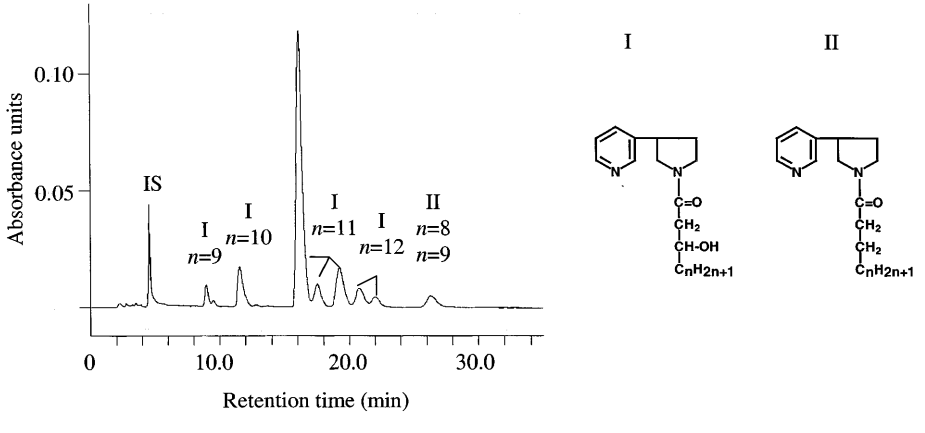

Fig. 1. Separation by HPLC of hydroxylated (I) and nonhydroxylated NacNNs (II) in a leaf surface extract of *Nicotiana repanda*. Compounds eluted in the following order: *IS* (2,2′-pyridil; internal standard); *n = 9* (N-hydroxydodecanoic acid nornicotine); *n = 10* (N-hydroxytridecanoic acid nornicotine); *n = 11* (N-hydroxytetradecanoic acid nornicotine); *n = 12* (N-hydroxypentanoic acid nornicotine); *n = 8* (N-undecanoic acid nornicotine); *n = 9* (N-dodecanoic acid nornicotine)

0.5 μm; Restek Bad Soden, Germany). The injector was maintained at 250 °C and the oven was programmed as follows: 50 °C (1 min), 20 °C min^{-1} to 170 °C, 10 °C min^{-1} to 210 °C, 20 °C min^{-1} to 250 °C (10 min). The mass spectrometer was scanned from 65 to 170 amu with 5.5 scans s^{-1}. The ions at 119, 120 and 121 (nornicotine) and at 133, 134, 135 amu (nicotine and anabasine) were chosen for the determination of the isotope enrichment corresponding to the $[M-29]^+$ fragment (Budzikiewicz et al. 1964). As reference, a dilution series of nicotine, nornicotine and anabasine was used.

Statistical analysis. One-way and repeated measures analysis of variance (ANOVA) as well as Fischer's protected least significant difference (PLSD) test within the ANOVA (Statview 4.5; Abacus Concepts, Berkeley, Calif., USA) were used to analyze the data.

Treatment of roots or shoots with MeJA increased the NacNN pools 1.5-fold (6 h, 12 h) to 2.17 fold (24 h, 48 h; Fig. 2). Four days after wounding leaves, NacNN pools were 4-fold higher in shoots of wounded plants than in unwounded controls (data not shown). These increases in shoot NacNN pools were highly significant (Fig. 2; repeated measures ANOVA $F_{2,30} = 17.758$; $P = 0.0005$). Interestingly, the increase was as rapid in plants that had MeJA applied to their roots as it was in plants receiving leaf treatments ($F_{1,21} = 0.195$; $P = 0.6718$). These results indicate that the rapid acylation of nornicotine is systemically activated and does not require wounding or direct stimulation of the leaves or trichomes for its activation.

This rapid (< 6 h) increase in NacNN pools contrasts with the slower induction of shoot nornicotine pools, which began to increase at 24 h (1.3-fold) and attained 1.7-fold increases by 48 h (Fig. 2). Both the differences between treatments in nornicotine pools (repeated measures ANOVA $F_{2,36} = 4.996$; $P = 0.0264$) and the change over harvests ($F_{3,36} = 13.799$; $P < 0.0001$) were statistically significant. From these results, we conclude that the jasmonate-induced increases in NacNN pools precede induced increases in the nornicotine pools.

The incorporation of ^{15}N into alkaloids is consistent with the biosynthetic scheme first proposed by Jones and co-workers (Zador and Jones 1986; Huesing et al. 1989). The absolute quantities of ^{15}N in the various alkaloids dramatically illustrate the biosynthetic flux of N among nicotine in the roots and nornicotine and NacNNs in the shoots (Fig. 3). The rapid increase in the labeled nicotine pool in the roots occurs approximately 1 d before a comparable increase in shoot nornicotine pools (Fig. 3), which is consistent with the de-novo synthesis of nicotine from $^{15}NO_3^-$ in the roots and its subsequent demethylation to nornicotine after a delay due to transport to the shoot. The relative dynamics of the ^{15}N-labeled nornicotine and NacNN pools in the shoot are comparable (Fig. 3), suggesting that these pools were rapidly equilibrating (Fig. 3). However, the rate of increase of ^{15}N in the NacNN pools (43 μg g^{-1} d^{-1}) was significantly higher than that of the nornicotine pool (8 μg g^{-1} d^{-1}) demonstrating that the flux of ^{15}N into NacNNs was highly favored (Fig. 3). The sizes of the ^{15}N-labeled pools on day 3 reflect the differences in the flux: shoot nornicotine pools (24 μg ^{15}N-nornicotine g^{-1}) were approximately one quarter the size of the NacNN pools (130 μg ^{15}N-NacNNs g^{-1}). We estimate that by day 3, plants had used 3.12% of their acquired ^{15}N in the biosynthesis of nicotine (0.93%), nornicotine

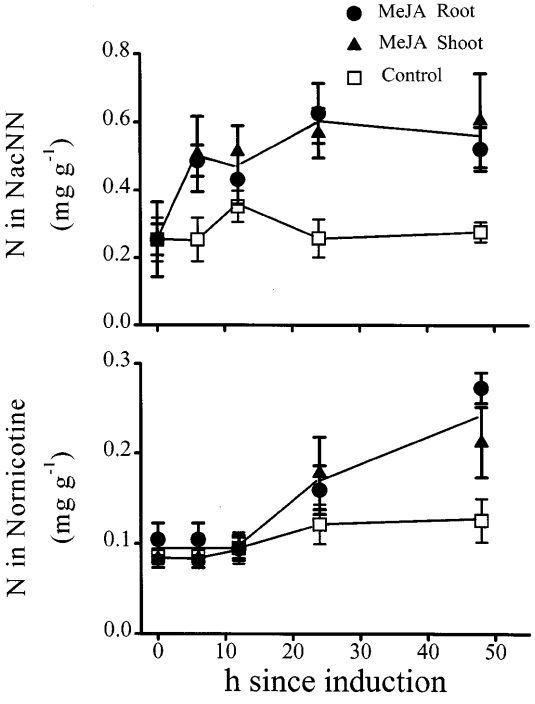

Fig. 2. Mean (±SE) N in NacNNs and nornicotine in the shoots of five replicate *N. repanda* plants harvested 6, 12, 24, 48 h after induction with MeJA. Plants were induced by application of MeJA either to the roots (250 μg, ●) or to the leaf surface (500 μg, ▲; see experiment protocol 2, *Materials and methods*). □, Control plants

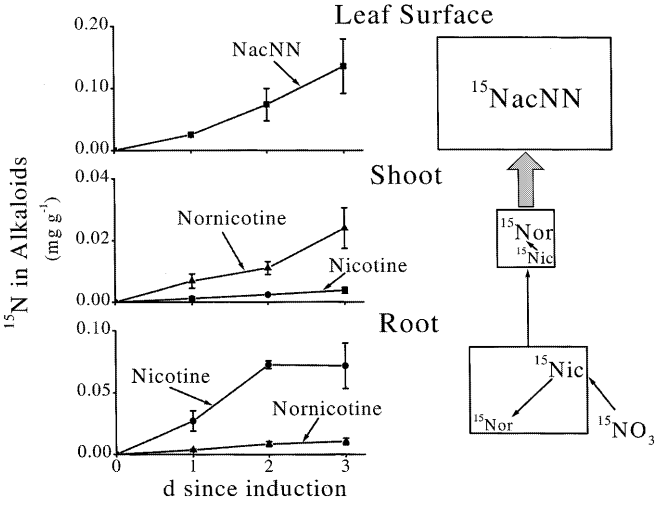

Fig. 3. Mean (±SE) ^{15}N in nicotine (●), nornicotine (▲) and NacNNs (■) in roots, shoots and on leaf surfaces of five replicate MeJA-induced (250 μg applied to the roots; see experiment protocol 1, *Materials and methods*) *N. repanda* plants harvested on days 1 to 3 after induction (*left*). Flux and pool sizes of ^{15}N from $^{15}NO_3^-$ into in the major alkaloid pools of the roots, shoots and leaf surface (*right*); *box sizes* reflect pool sizes on day 3 and *arrow thicknesses* reflect relative flux between compartments

(0.32%) and NacNNs (1.73%). In contrast, we estimate that only 0.14% of their acquired ^{15}N was used in the synthesis of anabasine by this time. Interestingly, while the rate of labeling of the anabasine pool was comparable to that of the other alkaloids, the flux into this pool was one-twentieth of that into the other alkaloids. Moreover, while the ^{15}N-labeled nicotine and nornicotine pools were significantly increased by jasmonate treatment (repeated measures ANOVA nicotine: $F_{1,18} = 192.888$, $P < 0.0001$; nornicotine: $F_{1,18} = 29.054$, $P = 0.0017$), the ^{15}N-labeled anabasine pool was not ($F_{1,18} = 0.322$; $P = 0.5854$). We conclude that unlike nicotine, nornicotine and the NacNNs, the flux of N into anabasine biosynthesis is small and not induced in this species.

The analysis of ^{15}N-labeled alkaloid pools in plants harvested throughout reproductive maturity and senescence illustrates one of the potential drawbacks of externalizing lipophilic alkaloids to the leaf surface, namely the difficulty of re-allocating this investment in defense to other parts as tissues senesce. On day 5, the ^{15}N-labeled NacNNs were evenly distributed among all leaf classes. However, by day 13 most of the ^{15}N-labeled NacNNs were located on senescing leaves (38% of total pools) and by day 22, 61% of the ^{15}N-labeled NacNN pools was found on dead and senescing leaves. In contrast, *N. sylvestris* produces large quantities of nicotine and re-allocates this large investment of N to younger leaves and reproductive parts – tissues with high fitness value – as the plant ages (Ohnmeiss and Baldwin 2000).

Here we provide the first demonstration that a trichome-based defense is rapidly induced. Trichomes are known to contain potent, but constitutively deployed, defenses. The observation that some of these may be inducibly deployed focuses attention on the costs and benefits of the rapid chemical changes on the plant surface, the first interface of plant-herbivore interactions. The ability to rapidly deploy chemicals on the leaf surface may help a plant optimize its defensive allocation, particularly if externalizing secondary metabolites limits a plant's ability to re-deploy the metabolite to other tissues. On the other hand, not all induced changes in surface chemistry are beneficial to plants. For example, the jasmonate-elicited increase in furanocoumarins on the leaf surface of *Apium graveolens* functions as an oviposition stimulant of the carrot fly. (Stanjek et al. 1997) Clearly, more attention needs to given to trichome-associated responses in plant-herbivore interactions.

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