Rapid HPLC Screening of Jasmonate-Induced Increases in Tobacco Alkaloids, Phenolics, and Diterpene Glycosides in *Nicotiana attenuata*

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A rapid, HPLC-based screening procedure for the main classes of secondary metabolites in *Nicotiana attenuata* leaves (alkaloids, phenolics, and diterpene glycosides) is reported. In a single step, leaves are extracted in aqueous acidified (0.5% acetic acid) methanol, and the extracted compounds are separated by reversed-phase HPLC with an acidic water/acetonitrile gradient in <30 min. The utility of the method in quantifying changes in the secondary metabolites after methyl jasmonate treatment of the plants, a treatment known to elicit resistance to herbivores in nature, is illustrated. Methyl jasmonate treatment elicited dramatic increases in some secondary metabolites (caffeoylputrescine, nicotine, and diterpene glycosides increased 12.5-, 1.4-, and 1.9-fold, respectively) but left others, such as rutin, unchanged. Such broad-based analytical screens will help characterize environmental and genetic changes in secondary metabolite profiles.

Keywords: *Nicotiana; tobacco; tobacco alkaloids; nicotine; phenolics; conjugated polyamines; diterpene glycosides; liquid chromatography; mass spectrometry; jasmonic acid*

INTRODUCTION

Nicotiana (Solanaceae) species contain a diverse array of secondary metabolites, the most important groups of which are the alkaloids, phenolic compounds, and terpenoids (*1*−*4*). These secondary metabolites mediate many of the biological interactions that the *Nicotiana* genus has with its environment (including a long-standing, addictive relationship with humans) and are produced by three different biosynthetic pathways. It is abundantly clear that the production of these metabolites is under strong environmental and genetic control, and nicotine is perhaps the best studied in this regard (*5*).

Different genotypes of cultivated tobacco (*N. tabacum*) are known to vary in their ability to accumulate and store nicotine, and in the native species, *N. sylvestris* and *N. attenuata*, wounding and herbivore attack are known to dramatically increase de novo nicotine synthesis and accumulation (*6*, *7*). These wound-induced increases can be elicited by treatment with the wound hormone, jasmonic acid, which functions as a signal mediating changes in secondary metabolism in response to many abiotic and biotic stresses (reviewed in ref *8*). Jasmonate treatment induces the accumulation of many secondary metabolites in plants or plant cell cultures, including alkaloids (*9*, *10*), terpenes (*11*), and phenolics (*12*). In *N. attenuata*, jasmonate treatment induces the accumulation of nicotine in the whole plant (*6*, *13*) and elicits durable herbivore resistance in nature (*14*), suggesting that nicotine contributes to jasmonate-induced resistance.

Most researchers studying environmental or genetic effects on secondary metabolite production tend to focus on a particular class of compounds for practical reasons; analyzing several groups of secondary compounds of various chemical properties is often time-consuming and requires the use of different extraction procedures and chromatographic methods. However, it is becoming abundantly clear that many different secondary metabolic pathways are coordinately regulated by biotic elicitors and environmental stresses (*15*). How these large-scale coordinated changes occur remains largely unknown, but the recent discovery of the ORCA-3 transcription factor, which regulates the expression of several genes in the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* (*16*), suggests that parts of secondary metabolism are under transcriptional control. As the functional analysis of gene products becomes a greater priority for researchers, analytical chemists will be asked to provide rapid screens of many different classes of metabolites. Such broad-based screens will be useful in studies of the chemical ecology of plants and in understanding the consequences of single gene transformations for metabolism. Here, we present such a broad-based screen, a simultaneous extraction procedure and HPLC analysis of alkaloids, phenolics, and diterpene glycosides in *N. attenuata*, and use the method to characterize jasmonate induced changes.

MATERIALS AND METHODS

Plant Material and Treatment with Methyl Jasmonate. *N. attenuata* Torr. ex Wats. plants from an inbred line originating from a natural population from Utah (*14*) were grown for 3 weeks as previously described (*6*). Rosette plants of a similar size and number of mature leaves (six to eight) were chosen for the experiment and divided randomly into treatment and control groups, 10 plants each. For each plant, we determined the source/sink transition leaf, which had just

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attained full expansion (see ref *11*). In a phyllotactic leaf sequence, the leaf one position older than the source/sink transition leaf was sprayed with methyl jasmonate dispersed in distilled water, resulting in 50 μg of methyl jasmonate per treated leaf. Four days later, the treated leaf and a younger leaf was harvested and analyzed for secondary compounds.

Extraction. A leaf half without a midvein (~100−200 mg) was harvested and frozen in liquid N_2. Leaf material was ground frozen in a 2 mL microcentrifuge tube. The samples were extracted by shaking the tubes vigorously for 2 h with 1.5 mL of 40% aqueous MeOH, containing 0.5% acetic acid. After centrifugation (12 min, 13000 rpm), the supernatant was transferred into a sample vial for HPLC analysis.

Standards and Solvents. Nicotine, nornicotine, anabasine, chlorogenic acid (5-caffeoylquinic acid), caffeic acid, putrescine, rutin (quercetin 3-rutinoside), scopoletin, and methyl jasmonate were from Sigma and Aldrich. Acetonitrile and methanol were from Merck.

HPLC Analysis. A Hewlett-Packard (Avondale, PA) HP 1100 series instrument with a binary pump, a vacuum degasser, an autosampler, a thermostated column compartment, and a diode array detector was used. Three RP columns with different characteristics were tested: Inertsil ODS-3, 3 μm, 150 × 4.6 mm i.d. (GL Sciences, Tokyo, Japan); Aqua 3 μm C18, 150 × 4.6 mm i.d. (Phenomenex, Torrance, CA); and PLRP-S 100 Å, 5 μm, 150 × 4.6 mm i.d. (Polymer Laboratories, Darmstadt, Germany). The Inertsil column performed the best and was attached to a Phenomenex SecurityGuard C18 precolumn. The solvents were (A) 0.25% H_3PO_4 in water (pH 2.2) and (B) acetonitrile. The elution system was as follows: 0−6 min, 0−12% of B; 6−10 min, 12−18% of B; 10−30 min, 18−58% of B. The flow rate was 1 mL/min, the injection volume was 15 μL, and the column oven was set at 24 °C. The eluent was monitored at 210, 254, 320, and 365 nm.

Identification and Quantification of the Compounds. The retention times and UV−vis spectra of the compounds were compared with those of authentic reference compounds. Chlorogenic acid isomers were prepared by heating chlorogenic acid in a saturated solution of $NaHCO_3$ (*17*), to produce a mixture of 3-, 4-, and 5-caffeoylquinic acids (neochlorogenic acid, cryptochlorogenic acid, and chlorogenic acid, respectively). Identifications were confirmed by coelution with the prepared chlorogenic acid isomers and commercially available standards. Caffeoylputrescine was isolated by HPLC using the analytical method, modified by replacing the H_3PO_4 in solvent A with 0.01% trifluoroacetic acid (TFA). Chlorogenic acid isomers and caffeoylputrescine were quantified at 320 nm as equivalents of chlorogenic acid. Nicotine and rutin were quantified at 254 and 365 nm, respectively. The amounts of compounds **6**−**9** (Figure 1) are expressed as peak areas at 210 nm/g of fresh weight.

Liquid Chromatography—Mass Spectrometry. LC-MS was performed on a Hewlett-Packard HP 1100 HPLC coupled to a Micromass Quattro II tandem quadrupole mass spectrometer (geometry quadrupole-hexapole-quadrupole) equipped with either an atmospheric pressure chemical ionization (APCI) or an electrospray (ESI) source. The phosphoric acid in the mobile phase was replaced by trifluoracetic acid. In APCI positive ion mode, the corona pin was operated at 3.5 kV and the sample cone at 16 V. In ESI positive ion mode the capillary and cone voltages were 3.7 kV and 25 V, respectively, whereas in ESI negative ion mode the capillary and cone voltages were 3.8 kV and 35 V, respectively. For APCI operation, vaporization was achieved with a nitrogen sheath gas (300 L h^{-1}) and drying gas (150 L h^{-1}) at 400 °C. For ESI analysis, nitrogen was used as the nebulization (20 L h^{-1}) and drying gas (150 L h^{-1}, 250 °C). Standard mass spectra were measured using the first quadrupole analyzer only. Product ion (MS/MS) mass spectra were recorded by setting the first quadrupole to transmit the parent ion of interest and scanning the second quadrupole. Argon was used as the collision gas, for collision-induced dissociation (CID), at 2×10^{-3} mbar, and a collision energy of 10−30 eV was employed to achieve fragmentation.

RESULTS AND DISCUSSION

Extraction. *N. attenuata* leaves are known to contain nicotine and small amounts of other tobacco alkaloids, phenolic compounds, such as chlorogenic acid and rutin, and a number of other secondary metabolites, such as diterpene glycosides. In addition to nicotine, chlorogenic acid, and rutin, preliminary HPLC analyses of *N. attenuata* leaves treated with methyl jasmonate indicated the presence of relatively large amounts of unknown compounds (peaks 2, 7, 8, and 9 in Figure 1), which were identified as caffeoylputrescine and diterpene glycosides. Because our aim was to rapidly screen as wide a range of secondary compounds as possible from the plant, the first task was to develop an extraction procedure suitable for the purpose. Phenolic compounds are most commonly extracted with aqueous alcohols (*18*), whereas for tobacco alkaloids, either basic or acidic aqueous solvents are typically used (*1*, *19*−*21*). Comparison of several extraction solvents, including acidified and nonacidified aqueous methanol, indicated that (1) aqueous methanol extracts all of the compounds better than 100% methanol; (2) acidic solvents are required for the extraction of caffeoylputrescine, but even small amounts of HCl are apparently deleterious for diterpene glycosides; (3) 0.5% acetic acid does not hydrolyze diterpene glycosides but does enhance the extraction of nicotine and caffeoylputrescine; and (4) the best overall extraction was achieved with 40−80% methanol acidified with 0.5% acetic acid, higher methanol contents favoring phenolic extraction. Comparison with samples extracted with 80% methanol, a commonly used extraction solvent for phenolics (*18*), indicated that all of the major phenolics present in *N. attenuata* leaves are detected by 40% methanol acidified with 0.5% acetic acid. Neochlorogenic acid and scopoletin, known constituents of other *Nicotiana* species (*3*), were not detected. However, due to the lower methanol content, the extraction efficiency is lower for lipophilic phenolics, such as scopoletin. Because our aim was to use the raw extract in the HPLC analysis without further purification steps, we wanted to use as low an organic content as possible, to avoid excessive extraction of lipophilic contaminants, and to achieve better compatibility between extraction solvent and the nonorganic starting eluent in HPLC. Therefore, 40% methanol with 0.5% acetic acid was chosen as the best compromise for fresh plant material, whereas for dry material, or *Nicotiana* species known to contain lipophilic phenolics, a higher methanol concentration may be used.

Chromatographic Conditions. Analyzing all of the desired compounds with the same solvent system raised an apparent problem: nicotine is often analyzed as a free base with a mobile phase buffered to a relatively high pH (6.25−7.4), because in acidic conditions (pH 2−4) it exists as a mixture of its mono- and diprotonated forms and is unretained. Conversely, at high pH, phenolic compounds are not well retained and the peaks are severely asymmetrical. However, it has been reported that certain reversed-phase (RP) columns give reasonable retention for nicotine when elution solvents buffered to pH 3.0 are used (*22*). Extracting and analyzing nicotine under acidic conditions has several potential advantages: the protonated form of nicotine is more stable and less volatile than its unprotonated form (*23*), and the UV absorbance of nicotine is more intense under acidic conditions. According to Yang et al. (*20*), the chromatographic peak area obtained for nicotine at pH

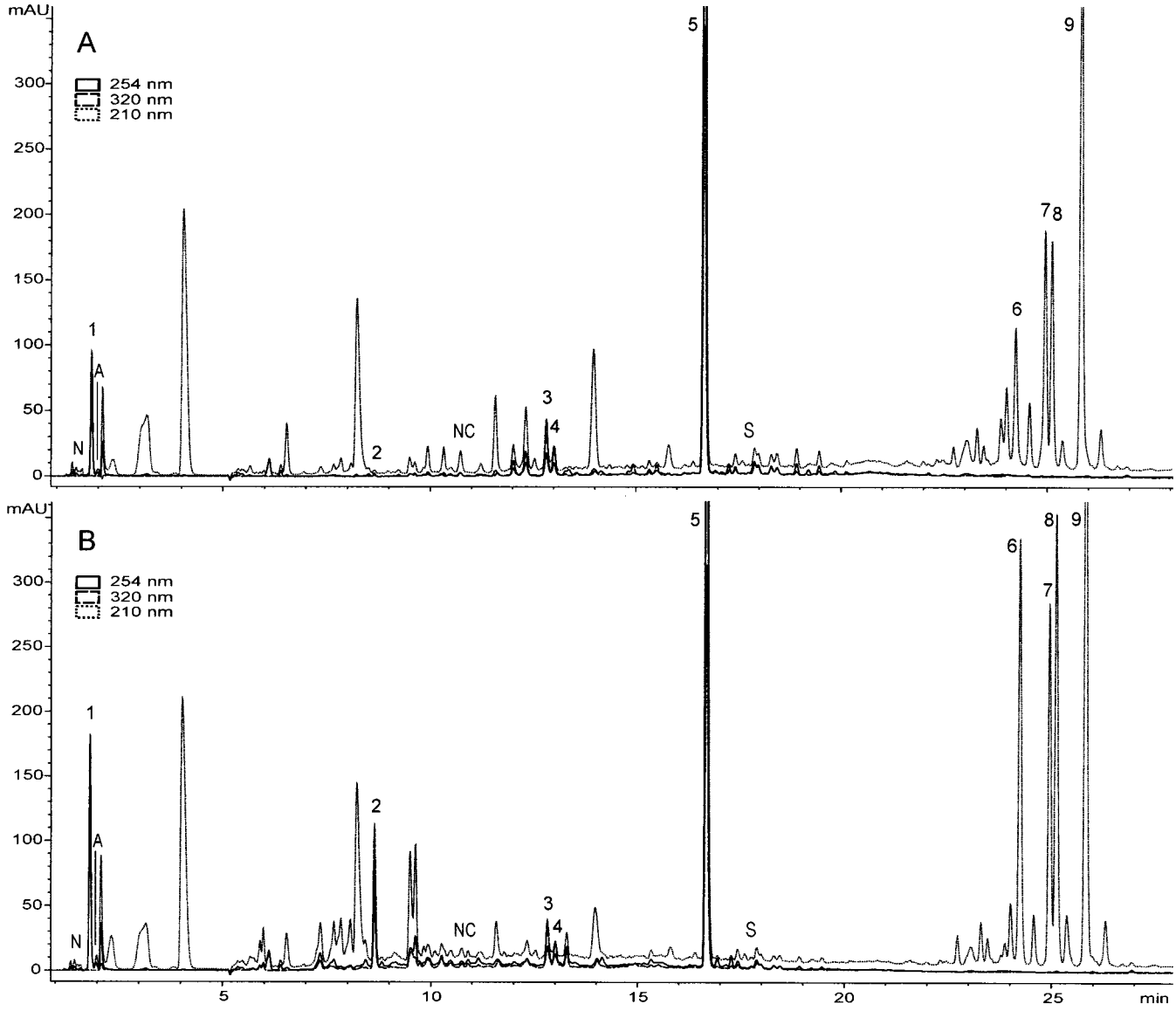

Figure 1. HPLC chromatograms of *N. attenuata* leaves ("treated leaf", i.e., the leaf one position older than the source/sink transition leaf): (A) control; (B) methyl jasmonate treatment. Peaks: (1) nicotine, (2) caffeoylputrescine, (3) chlorogenic acid, (4) cryptochlorogenic acid, (5) rutin, (6−9) diterpene glycosides. N, A, NC, and S indicate the retention times of the standard compounds nornicotine, anabasine, neochlorogenic acid, and scopoletin, respectively.

>7 is approximately half of that obtained at pH 2.5. Moreover, the peak shapes for nicotine and various other basic compounds are more symmetrical under acidic conditions (*22*, *24*, *25*).

We found that a gradient elution based on acetonitrile, starting from water acidified with 0.25% orthophosphoric acid, allowed the separation of the desired compounds but with little retention for tobacco alkaloids. We decided not to use solvents buffered with salts but rather to acidify them with orthophosphoric acid, because the analysis required a gradient run to relatively high concentration of organic solvent, which could precipitate salts.

The analysis of nicotine was found to be critical for the method and, therefore, we compared three RP columns with different characteristics: Inertsil ODS-3, Aqua 3 μm, and PLRP-S 100 Å. Inertsil ODS columns have been found to be suitable for the analysis of nicotine at acidic conditions (*22*). Aqua is end-capped with a hydrophilic reagent, which the manufacturer claims to give better performance under aqueous conditions and different selectivity for polar compounds than ordinary C18 columns. PLRP-S is a highly retentive cross-linked copolymer of styrene and divinylbenzene (PS/DVB), which provides better chemical stability than bonded C18 phases.

Authentic samples of nicotine, nornicotine, and anabasine, as well raw extracts from *N. attenuata*, were analyzed using the analytical HPLC method as described. The Inertsil ODS-3 was the only column to give sufficient retention for tobacco alkaloids and a good separation of all the compounds analyzed. The PLRP-S column gave less separation for most of the compounds, whereas the Aqua column failed completely in the analysis of tobacco alkaloids. Nicotine, nornicotine, and anabasine peaks were all severely distorted, each giving two tailing peaks with a broad peak between connecting the two.

Identification of the Compounds. The identity of caffeoylputrescine was established by LC-MS. APCI analysis produced a molecular species from a purified sample ($[M + H]^+$) at *m*/*z* 251 (accurate mass: measured

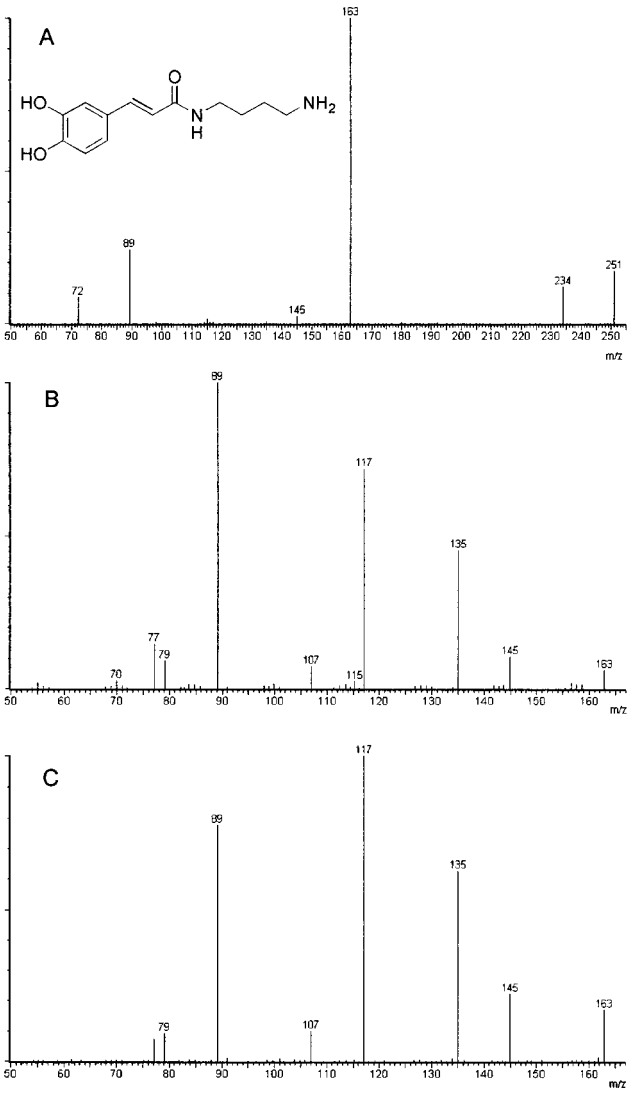

Figure 2. Low-energy CID mass spectra of (A) $[M + H]^+$ from compound **2** (caffeoylputrescine), (B) *m*/*z* 163 from compound **2**, and (C) *m*/*z* 163 from caffeic acid.

251.1417, calculated for $C_{13}H_{19}N_2O_3$ 251.1396) that, upon low-energy CID, fragmented to yield *m*/*z* 163 as the major product ion (Figure 2A). We suspected *m*/*z* 163 to be the acylium ion from a caffeoyl moiety, and this was confirmed by comparison of the CID product ion spectrum of the 163 ion, from **2**, with that from authentic caffeic acid (Figure 2B,C). From the molecular formula, the remainder of the molecule was composed of $C_4H_{11}N_2$, and comparison of CID products from the ion at *m*/*z* 89 (Figure 2A) with those of $[M + H]^+$ from authentic putrescine gave a good match, identifying **2** as caffeoylputrescine.

On the basis of APCI and ESI MS and MS/MS, the suspected glycosides **6**−**9** were identified as conjugates of geranyllinalool (*4*). CID fragmentation of the molecular species yielded an aglycon core with a mass of 306 (consistent with geranyllinalool). Dissociation of this ion ($[M + H] = m/z$ 307) gave two losses of H_2O and a series of low-mass ions characteristic for isoprenoids (69, 81, 95, 109, 121, 149, and 163). The carbohydrate residues were isobaric with glucose (glc) and rhamnose (rha) and, because glucopyranosylrhamnopyranosyl conjugates of geranyllinalool are known in *Nicotiana* (*4*), most probably were these sugars: **6** contained two glc, **7** and **8** contained one glc and one rha, and **9** contained one glc and three rha.

Table 1. Concentrations[a] of Secondary Compounds in *N. attenuata* Leaves after Methyl Jasmonate Treatment[b]

compound (peak no.)	control	methyl jasmonate	increase	*P*
	Treated Leaf			
nicotine (1)	193 ± 18.6	264 ± 18.4	1.4×	*
caffeoylputrescine (2)	5.2 ± 1.02	65 ± 13.8	12.5×	***
chlorogenic acid (3)	27 ± 4.0	45 ± 5.0	1.7×	**
cryptochlorogenic acid (4)	15.4 ± 2.11	22.2 ± 2.4	1.4×	*
rutin (5)	465 ± 14.8	508 ± 29.9	1.1×	ns
DTG6 (6)	4.7 ± 0.48	11.3 ± 0.68	2.4×	***
DTG7 (7)	6.2 ± 0.88	8.9 ± 0.57	1.4×	***
DTG8 (8)	5.2 ± 0.30	12.3 ± 0.86	2.4×	***
DTG9 (9)	13.7 ± 1.28	23.4 ± 1.98	1.7×	***
total DTG	29.7 ± 1.67	55.9 ± 3.57	1.9×	***
	Younger Leaf			
nicotine	244 ± 17.1	421 ± 32.4	1.7×	***
caffeoylputrescine	6.6 ± 2.10	33 ± 6.9	5.0×	**
chlorogenic acid	27 ± 8.9	31 ± 9.4	1.1×	ns
cryptochlorogenic acid	14 ± 4.6	15 ± 4.4	1.1×	ns
rutin	691 ± 45.7	711 ± 36.6	1.0×	ns
DTG6	8.4 ± 0.69	12.9 ± 0.68	1.5×	***
DTG7	9.6 ± 0.59	12.1 ± 0.36	1.3×	**
DTG8	9.0 ± 0.71	14.7 ± 0.80	1.6×	***
DTG9	19.8 ± 2.00	29.6 ± 1.33	1.5×	***
total DTG	46.8 ± 3.05	69.2 ± 2.10	1.5×	***

[a] Expressed as μg/g of fresh weight; caffeoylputrescine and cryptochlorogenic acid expressed as chlorogenic acid; diterpene glycosides (DTG) expressed as peak areas at 210 nm/mg fresh weight. [b] Data are means ± SE, $n = 10$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Methyl Jasmonate Treatment. The concentrations of all the analyzed compounds were higher in plants treated with methyl jasmonate (Table 1), and the only compound not to show a significant increase in either the treated or untreated leaf was rutin. The most striking change was seen in the concentration of caffeoylputrescine, which increased 12.5-fold in the leaves treated with methyl jasmonate. Nicotine and diterpene glycoside concentrations in treated leaves were 1.4- and 1.9-fold higher, respectively, than in controls. In the untreated, younger leaves, responses were mostly similar, except for a slightly larger (1.7-fold) increase in nicotine and a smaller increase for caffeoylputrescine (6-fold). Chlorogenic acid isomers showed an increase in the treated leaves but no change in untreated leaves.

Caffeoylputrescine was originally isolated as a new natural product from callus tissue culture of *N. tabacum* (*26*). Later, it was reported from apical leaves, anthers, and ovaries of flowering tobacco plants (*27*, *28*). Although hydroxycinnamic acid amides reported from higher plants have often been associated with reproductive parts of the plant, they have also been implicated in inducible defense systems. For example, the levels of caffeoylputrescine, and other hydroxycinnamic acid amides, are known to increase in tobacco leaves after ozone exposure (*29*) and in elicitor-treated cell suspension cultures and fungus-infected leaves of potato (*Solanum tuberosum* cv. Datura; *30*). In potato cell cultures, the concentration of caffeoylputrescine decreased rapidly after 24 h and was almost at the basal level at 48 h. Moreover, in barley, the production of *p*-coumaroyl- and feruloylagmatines, precursors of antifungal hordatines, have been shown to be induced by fungal infection (*31*) and jasmonate treatment (*12*). Accumulation of hydroxycinnamic acid amides by jasmonates has also been reported in red clover (*32*) and

in root cultures of *Hyoscyamus muticus* (*33*), whereas in tomato leaves, the levels of feruloyl- and *p*-coumaroyltyramine increased in response to wounding and treatment with chitosan but not after jasmonate treatment (*34*).

In *N. attenuata*, the concentration of caffeoylputrescine is strongly induced by methyl jasmonate, clearly increased already after 10−30 h after treatment, and is at the same level or lower at 100 h, whereas it takes 4−5 days for nicotine to increase (*6*). Therefore, our choice of harvesting 4 days after methyl jasmonate treatment may have underestimated the changes taking place in the leaves. Except for nicotine, all of the changes were greater in the leaf treated with methyl jasmonate. This is most likely explained by the mechanism of induced nicotine accumulation. The biosynthesis of nicotine takes place in the roots and, therefore, requires a signal from the leaves and transport of the newly synthesized nicotine to leaves. However, nicotine is transported preferably to younger leaves (*35*), apparently because they are the most valuable leaves to defend. All of the other compounds are likely to be synthesized in the leaves, and their increase in the untreated leaves would indicate a systemic response. However, because we sprayed the leaves with methyl jasmonate, we cannot exclude the possibility that the untreated leaves received some of the droplets or received methyl jasmonate in gaseous form during the treatment.

Although we found a significant increase in chlorogenic acid isomers after methyl jasmonate treatment in this study, this appears not to happen consistently in *N. attenuata* or *N. sylvestris* (M. Keinänen, C. A. Preston, and I. T. Baldwin, unpublished data). Chlorogenic acid isomers and rutin concentrations also often show larger variation than nicotine or diterpene glycosides in *N. attenuata*. These phenolics are at least partly present in the glandular hairs on the leaf surfaces, which also contain polyphenol oxidase (PPO) activity. These hairs are easily broken upon touching, which may then lead to the oxidation of the phenolics and, thus, may increase variation. However, rutin concentrations were not significantly induced (Table 1), which suggests that the observed differences were not due to sample handling.

Snook et al. (*4*) reported that leaves of tobacco introduction TI-165 resistant to tobacco budworm (*Heliothis virescens* F.) contained relatively high levels of 16-hydroxygeranyllinalool glycosides that were absent in susceptible varieties. Bioassays with tobacco leaves and isolated compounds showed significant negative correlation with larval growth, which indicates that these diterpene glycosides contribute to budworm resistance of tobacco plants. Furthermore, HPLC analyses of 68 *Nicotiana* species indicated that 26 species, including *N. attenuata*, had high levels (>2.5% dry weight) of identical or related diterpene glycosides. In accordance with their results, we have found high levels of related compounds in *N. attenuata* (this study), intermediate levels in *N. tabacum*, and none in *N. sylvestris* (M. Keinänen and I. T. Baldwin, unpublished data).

On the basis of cell culture studies, Gundlach et al. (*9*) showed that jasmonate treatment induced a wide range of secondary compounds in several plant species and suggested that induction by jasmonate may not be restricted to any specific type of secondary compound, but may rather be a more or less general response. Indeed, there are now many reports of different secondary compounds being induced by jasmonates in various unrelated plants but apparently no data on the effect of jasmonates on a range of secondary compounds produced via different pathways in one plant species. Although our results show a rather general induction of secondary metabolites after methyl jasmonate treatment in *N. attenuata* leaves, there were also clear differences among compounds. In comparison to the other compounds analyzed, caffeoylputrescine showed a dramatic increase in both treated and untreated leaves, whereas the major flavonoid present, rutin, was not induced, and some of the unidentified peaks in the chromatograms (Figure 1) showed a tendency to decrease after jasmonate treatment (results not shown).

In conclusion, we present a relatively rapid and simple method for the simultaneous analysis of the three major groups of secondary compounds present in *N. attenuata*, most of which have been shown to have some activity against herbivores or pathogens of *Nicotiana* species. This method makes it possible to rapidly screen for changes in the secondary compound profile of the plants in ecological or physiological studies and to detect changes in the chemical composition in transgenic plants, which may well be unexpected. As an example, we have used the method to simultaneously detect changes in the three major groups of secondary compounds in the leaves of *N. attenuata* after methyl jasmonate treatment.

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